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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/080,522	02/25/2002	Russel E. Kaufman	1579-645	8638

23117 7590 10/25/2004

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EXAMINER	
HELMS, LARRY RONALD	
ART UNIT	PAPER NUMBER
1642	

DATE MAILED: 10/25/2004

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/080,522	KAUFMAN ET AL.	
	Examiner	Art Unit	
	Larry R. Helms	1642	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *26 July 2004*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-32* is/are pending in the application.

4a) Of the above claim(s) *1-20,23-28,31 and 32* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *21,22,29 and 30* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☒ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *06 January 2003* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date *2/25/02*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

1. Applicant's election with traverse of Group III, claims 21-22, 29-30 in the reply filed on 7/26/04 is acknowledged. The traversal is on the ground(s) that Groups III and V should be examined together and no undue burden would be placed on the examiner. This is not found persuasive because as stated in the restriction requirement the groups are distinct based on classification as well as the fact that the antibody can be used for a different method as stated in the restriction requirement. Therefore, the restriction is maintained and it is acknowledged that Group V can be rejoined when Group III claims are allowable. Applicants attention is directed to paragraph 4 of the restriction requirement for rejoinder practice.

The requirement is still deemed proper and is therefore made FINAL.

2. Claims 1-20, 23-28, 31-32 are withdrawn from further consideration pursuant to 37 CFR 1.142(b), as being drawn to a nonelected inventions, there being no allowable generic or linking claim. Applicant timely traversed the restriction (election) requirement in the reply filed on 7/26/04.

3. Claims 21-22 and 29-30 are under examination.

Specification

4. The disclosure is objected to because of the following informalities:

a. The first line of the specification needs to be updated to indicate application 09/539,774 is now US Patent 6,350,615.

b. The title of the invention is not descriptive. A new title is required that is clearly indicative of the invention to which the claims are directed.

c. Although this application appears to be in sequence compliance, it is requested that either the Figures or the Brief Description of the Drawings include any SEQ ID NOs that are in the Figures.

d. The ATCC address needs to be updated on page 15 to the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209

Appropriate correction is required.

Claim Objections

5. Claims 21-22 are objected to because of the following informalities: Claims 21 and 22 depend on non-elected claim 19. For examination all of the dependencies of claim 19 will be read into claim 21 and 22.

Appropriate correction is required.

Claim Rejections - 35 USC § 112

6. The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the

art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

7. Claims 21-22, 29-30 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

The claims encompass an antibody to any mammalian K12 protein or any compound that binds to K12 protein. There is insufficient written description encompassing "mammalian K12 protein" because the relevant identifying characteristics of the genus of such a structure is not given except for SEQ ID NO:1. In addition the only "compound that specifically binds to K12" is an antibody to SEQ ID NO:1. The identifying characteristics of the mammalian K12 protein or other physical and/or chemical characteristics of the protein are not set forth in the specification as-filed, commensurate in scope with the claimed invention. Vas-Cath Inc. v. Mahurkar, 19 USPQ2d 1111, makes clear that "applicant must convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession of the invention. The invention is, for purposes of the 'written description' inquiry, whatever is now claimed." (see page 1117). The specification does not "clearly allow persons of ordinary skill in the art to recognize that [he or she] invented what is claimed." (see Vas-Cath at page 1116).

Adequate written description requires more than a mere statement that it is part of the invention and reference to a potential method for isolating it. See Fiers v. Revel,

25 USPQ2d 1601, 1606 (CAFC 1993) and Amgen Inc. v. Chugai Pharmaceutical Co. Ltd., 18 USPQ2d 1016.

One cannot describe what one has not conceived. See Fiddles v.Baird, 30 USPQ2d 1481, 1483. In Fiddles v. Baird, claims directed to mammalian FGF's were found unpatentable due to lack of written description for the broad class. The specification provided only the bovine sequence. Thus, the specification fails to describe these DNA sequences. The Court further elaborated that generic statements are not adequate written description of the genus because it does not distinguish the claimed genus from others, except by function. Finally, the Court indicated that while applicants are not required to disclose every species encompassed within a genus, the description of a genus is achieved by the recitation of a representative number of DNA molecules, defined by nucleotide sequence, falling within the scope of the genus, See The Reagents of the University of California v. Eli Lilly and Company, 43 USPQ2d 1398, 1406 (Fed. Cir. 1997).

Applicant is relying upon SEQ ID NO:1 as a species to support an entire genus of mammalian K12 protein for which applicant only had the human protein of SEQ ID NO:1. In addition the specification only teaches an antibody to SEQ ID NO:1 as a compound that binds. There is no other compound as broadly encompassed by small molecules, peptides, etc that are described in the specification that is a compound that binds.

With respect to a compound that would be suitable for use in the claimed invention, per the *Enzo* court's example of a description of an anti-inflammatory steroid

couched "in terms of its function of lessening inflammation of tissues," which, the court stated, "fails to distinguish any steroid from others having the same activity or function," and which therefore, fails to satisfy the written-description requirement. Similarly, "a compound that inhibits specific binding between a signal-transducing protein and a cytoplasmic protein..." does not distinguish the compound from others having the same activity or function and as such does not satisfy the written-description requirement. Mere idea or function is insufficient for written description; isolation and characterization at a minimum are required. The identity of the compound, and the description must convey what the compound is, and not just what it does. The instant application discloses no more than a hoped-for function (binding) for an as-yet-to-be-discovered compound.

The guidelines for the Examination of Patent Applications Under the 35 U.S.C. 112 first paragraph "written description" requirement make clear that if a claimed genus does not show actual reduction to practice for a representative number of species; then the requirement may be alternatively met by reduction to drawings, or by disclosure of relevant, identifying characteristics, i.e., structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the genus (Federal register, Vol. 66, No. 4, pages 1099-1111, Friday January 5, 2001, see especially page 1106 column 3).

In the absence of structural characteristics that are shared by members of the genus of mammalian K12 proteins or compounds that bind to K12; one of skill in the art would reasonably conclude that the disclosure fails to provide a representative number of species to describe the genus. Thus, Applicant was not in possession of the claimed genus. See University of California v. Eli Lilly and Co. 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997).

Applicant is reminded that Vas-Cath makes clear that the written description provision of 35 U.S.C. 112 is severable from its enablement provision (see page 1115).

8. Claim 22 is rejected under 35 U.S.C. § 112, first paragraph, because the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention, because the specification does not provide evidence that the claimed biological materials are (1) known and readily available to the public; (2) reproducible from the written description.

It is unclear if a cell line which produces an antibody having the exact chemical identity of 7C3 is known and publicly available, or can be reproducibly isolated without undue experimentation. Therefore, a suitable deposit for patent purposes is suggested. Without a publicly available deposit of the above cell line, one of ordinary skill in the art could not be assured of the ability to practice the invention as claimed. Exact replication of: (1) the claimed cell line; (2) a cell line which produces the chemically and functionally distinct antibody claimed; and/or (3) the claimed antibody's amino acid or nucleic acid sequence is an unpredictable event.

For example, very different VH chains (about 50% homologous) can combine with the same VK chain to produce antibody-binding sites with nearly the same size, shape, antigen specificity, and affinity. A similar phenomenon can also occur when different VH sequences combine with different VK sequences to produce antibodies with very similar properties. The results indicate that divergent variable region sequences, both in and out of the complementarity-determining regions, can be folded to form similar binding site contours, which result in similar immunochemical characteristics. [FUNDAMENTAL IMMUNOLOGY 242 (William E. Paul, M.D. ed., 3d ed. 1993)]. Therefore, it would require undue experimentation to reproduce the claimed antibody species 7C3. Deposit of the hybridoma would satisfy the enablement requirements of 35 U.S.C. § 112, first paragraph. See, 37 C.F.R. 1.801-1.809.

Applicants referral on page 15 that the hybridoma producing the 7C3 antibody has been deposited at the ATCC is not persuasive because all of the assurances have not been met.

If the deposit is made under the provisions of the Budapest Treaty, filing of an affidavit or declaration by applicant or assignees or a statement by an attorney of record who has authority and control over the conditions of deposit over his or her signature and registration number stating that the deposit has been accepted by an International Depository Authority under the provisions of the Budapest Treaty and that all restrictions upon public access to the deposited material will be irrevocably removed upon the grant of a patent on this application. This requirement is necessary when deposits are made

under the provisions of the Budapest Treaty as the Treaty leaves this specific matter to the discretion of each State.

If the deposit is not made under the provisions of the Budapest Treaty, then in order to certify that the deposits comply with the criteria set forth in 37 CFR 1.801-1.809 regarding availability and permanency of deposits, assurance of compliance is required. Such assurance may be in the form of an affidavit or declaration by applicants or assignees or in the form of a statement by an attorney of record who has the authority and control over the conditions of deposit over his or her signature and registration number averring:

(a) during the pendency of this application, access to the deposits will be afforded to the Commissioner upon request:

(b) all restrictions upon the availability to the public of the deposited biological material will be irrevocably removed upon the granting of a patent on this application:

(c) the deposits will be maintained in a public depository for a period of at least thirty years from the date of deposit or for the enforceable life of the patent of or for a period of five years after the date of the most recent request for the furnishing of a sample of the deposited biological material, whichever is longest; and

(d) the deposits will be replaced if they should become nonviable or non-replicable.

Amendment of the specification to recite the date of deposit and the complete name and address of the depository is required. As an additional means for completing the record, applicant may submit a copy of the contract with the depository for deposit and maintenance of each deposit.

If a deposit is made after the effective filing date of the application for patent in the United States, a verified statement is required from a person in a position to

corroborate that the biological material described in the specification as filed is the same as that deposited in the depository, stating that the deposited material is identical to the biological material described in the specification and was in the applicant's possession at the time the application was filed.

Applicant's attention is directed to In re Lundak, 773 F.2d. 1216, 227 USPQ 90 (CAFC 1985) and 37 CFR 1.801-1.809 for further information concerning deposit practice.

9. Claims 21-22 and 29-30 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for an antibody that binds SEQ ID NO:1 or a fragment of at least 5 amino acids of SEQ IS NO:1 wherein the antibody can be 7C3, with completion of the deposit requirements, does not reasonably provide enablement for just any compound that binds to just any K12 protein or an antibody to just any mammalian K12 protein. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

Factors to be considered in determining whether undue experimentation is required, are summarized in Ex parte Forman, 230 USPQ 546 (BPAI 1986). They include the nature of the invention, the state of the prior art, the relative skill of those in the art, the amount of direction or guidance disclosed in the specification, the presence or absence of working examples, the predictability or unpredictability of the art, the

breadth of the claims, and the quantity of experimentation which would be required in order to practice the invention as claimed.

The claims are broadly drawn to an antibody or any compound that binds to just any K12 protein or a mammalian K12 protein.

The specification teaches only SEQ ID NO:1 as the human K12 protein and only an antibody as a compound that binds to SEQ ID NO:1 (see Figure 1 and Example IV)

The claims are not commensurate in scope with the enablement provided in the specification because the specification does not disclose a function of the K12 protein or what identifying characteristics would be needed for one to have a K12 protein. Although the amino acid sequence of SEQ ID NO:1 is disclosed, there's no indication as to what parts of the protein are required to have a "K12" protein. The structure of the protein is required for a function to be determined and as evidenced from the following discussion protein chemistry is probably one of the most unpredictable areas of biotechnology.

For example, the replacement of a single lysine at position 118 of the acidic fibroblast growth factor by a glutamic acid led to a substantial loss of heparin binding, receptor binding, and biological activity of the protein (see Burgess et al, Journal of Cell Biology Vol 111 November 1990 2129-2138). In transforming growth factor alpha, replacement of aspartic acid at position 47 with asparagine, did not affect biological activity while the replacement with serine or glutamic acid sharply reduced the biological activity of the mitogen (see Lazar et al Molecular and Cellular Biology Mar 1988 Vol 8 No 3 1247-1252). Replacement of the histidine at position 10 of the B-chain of human

insulin with aspartic acid converts the molecule into a superagonist with 5 times the activity of nature human insulin. Schwartz et al, Proc Natl Acad Sci USA Vol 84:6408-6411 (1987). Removal of the amino terminal histidine of glucagon substantially decreases the ability of the molecule to bind to its receptor and activate adenylate cyclase. Lin et al Biochemistry USA Vol 14:1559-1563 (1975).

These references demonstrate that even a single amino acid substitution or what appears to be an inconsequential chemical modification, will often dramatically affect the biological activity of the protein.

The claims encompass an antibody or any compound that binds to any K12 protein and as evidenced from the above discussion it would be unpredictable what other proteins are a K12 protein. In addition, the specification has not disclosed any other "compound" that would bind to SEQ ID NO:1 that could be used for detection of SEQ ID NO:1

Therefore, in view of the unpredictability in the art of protein chemistry as indicated above, and in view of the lack or guidance in the specification and in view of the broadly claimed invention, it would require undue experimentation to practice the broadly claimed invention.

Claim Rejections - 35 USC § 101

10. 35 U.S.C. 101 reads as follows:

> Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

11. Claims 21-22 are rejected under 35 U.S.C. ' 101 because the claimed invention

is directed to non-statutory subject matter.

Claims 21-22 as written, do not sufficiently distinguish over antibodies as they exists naturally because the claim does not particularly point out any non-naturally occurring differences between the claimed antibodies and binding compositions and the structure of naturally occurring antibodies.

In the absence of the hand of man, the naturally occurring antibodies are considered non-statutory subject matter (Diamond v. Chakrabarty, 206 U.S.P.Q. 193 (1980)). It should be noted that the mere purity of a naturally occurring product does not necessarily impart patentability (Ex parte Siddiqui, 156 U.S.P.Q. 426 (1966)). However, when purification results in a new utility, patentability is considered (Merck Co. v. Chase Chemical Co., 273 F.Supp 68 (1967), 155 USPQ 139, (District Court, New Jersey, 1967)). Amendment of the claim to recite "an isolated" or "purified" antibody or similar language would obviate this rejection.

Conclusion

12. No claim is allowed.

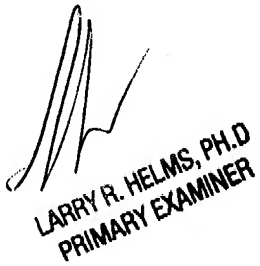
13. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Larry R. Helms, Ph.D, whose telephone number is (571) 272-0832. The examiner can normally be reached on Monday through Friday from 6:30 am to 4:00 pm, with alternate Fridays off. If attempts to reach the examiner by

telephone are unsuccessful, the examiner's supervisor, Jeffery Siew, can be reached at (571) 272-0787.

14. Papers related to this application may be submitted to Group 1600 by facsimile transmission. Papers should be faxed to Group 1600 via the PTO Fax Center. The faxing of such papers must conform with the notice published in the Official Gazette, 1096 OG 30 (November 15, 1989). The Fax Center telephone number is 703-872-9306.

Larry R. Helms

571-272-0832



Notice of References Cited	Application/Control No. 10/080,522	Applicant(s)/Patent Under Reexamination KAUFMAN ET AL.	
	Examiner Larry R. Helms	Art Unit 1642	Page 1 of 2

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
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	F	US-			
	G	US-			
	H	US-			
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	J	US-			
	K	US-			
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	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
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	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	FUNDAMENTAL IMMUNOLOGY 242 (William E. Paul, M.D. ed., 3d ed. 1993)
	V	Burgess et al, Journal of Cell Biology Vol 111 November 1990 2129-2138
	W	Lazar et al Molecular and Cellular Biology Mar 1988 Vol 8 No 3 1247-1252
	X	Schwartz et al, Proc Natl Acad Sci USA Vol 84:6408-6411 (1987

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Notice of References Cited	Application/Control No. 10/080,522	Applicant(s)/Patent Under Reexamination KAUFMAN ET AL.	
	Examiner Larry R. Helms	Art Unit 1642	Page 2 of 2

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	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Lin et al Biochemistry USA Vol 14:1559-1563 (1975
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Sheet 1 of 2

INFORMATION DISCLOSURE CITATION

(Use several sheets if necessary)

ATTY. DOCKET NO.: 1579-645

CONTINUATION OF APPLICATION SERIAL NO.: 09/539,774

APPLICANT: KAUFMAN et al

FILING DATE: February 25, 2002

GROUP:

JC858 U.S. PTO 10/080522 02/25/02

U.S. PATENT DOCUMENTS

*EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE

FOREIGN PATENT DOCUMENTS

		DOCUMENT	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO
LM		0 652 232	5/1995	EPO				
LM		WO 94/00601	1/1994	PCT				
LM		WO 94/00603	1/1994	PCT				
LM		WO 92/13970	8/1992	PCT				

OTHER DOCUMENTS (including Author, Title, Date, Pertinent pages, etc.)

*Examiner: [signature] Date Considered: 8/26/04

Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to application.

Form PTO-FB-A820 (Also PTO-1449)

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Sheet 2 f 2

INFORMATION DISCLOSURE CITATION

(Use several sheets if necessary)

ATTY. DOCKET NO.: 1579-645

CONTINUATION OF APPLICATION SERIAL NO.: 09/539,774

APPLICANT: KAUFMAN et al

FILING DATE: February 25, 2002

GROUP:

JC866 U.S. PTO 10/080522 02/25/02

U.S. PATENT DOCUMENTS

*EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
		5,589,358	12/1996	Dawson			

FOREIGN PATENT DOCUMENTS

		DOCUMENT	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent pages, etc.)

LM		Lee, "The Accumulation of Three Specific Proteins Related to Glucose-Regulated Proteins in a Temperature-Sensitive Hamster Mutant Cell Line K12", Journal of Cellular Physiology 106:119-125 (1981)
LM		Cole et al, "Cloning and Expression in *Escherichia coli* K-12 of the Genes for Major Outer Membrane Protein OmpA from *Shigella dysenteriae, Enterobacter aerogenes,* and *Serratia marcescens*", Journal of Bacteriology 149(1):145-150 (1982)
LM		Slentz-Kesler et al, "Identification and Characterization of K12 (SECTM1), a Novel Human Gene That Encodes a Golgi-Associated Protein with Transmembrane and Secreted Isoforms", Genomics 47:327-340 (1998)

*Examiner: [signature] Date Considered: 8/26/04

Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to application.

Form PTO-FB-A820 (Also PTO-1449)

elicited in response to each carbohydrate antigen, it is clear that a tremendous diversity of antibody molecules must be available, from which some antibodies can be selected for every possible antigenic structure. In order to regulate such a diverse system, a network theory has been proposed, in which antibodies are themselves recognized as antigenic (see Chapters 12 and 24, and ref. 19), and the response to streptococcal polysaccharide is a leading example in which anti-idiotypic antibodies can be shown to regulate the response to antigen (20).

Recent studies of a series of 17 monoclonal anti-α (1 → 6) dextran hybridomas (21,22) have investigated whether the binding sites of closely related antibodies would be derived from a small number of variable region genes, for both heavy and light chains, or whether antibodies of the same specificity could derive from variable region genes with highly divergent sequences. Each monoclonal had a groove-type site that could hold six or seven sugar residues (with one exception), based on inhibition of immunoprecipitation by different length oligosaccharides. Thus, unlike monoclonals to haptenated proteins, the precise epitope could be well characterized and was generally quite similar among the entire series.

Studies of the V_κ sequences revealed that only three V_κ groups were used in these hybridomas. Use of each V_κ group correlated with the particular antigen used to immunize the animals, whether linear dextran or short oligosaccharides, so that 10 of the monoclonals from mice immunized the same way all used the same V_κ.

In contrast, the 17 V_H chains were derived from at least five different germline genes from three different V_H gene families (23). The two most frequently used germline V_H genes were found in seven and five monoclonals, respectively, with minor variations explainable by somatic mutations. Once again, V_H gene usage correlated with size of the antigen used to immunize, although the length of each CDR did not correlate with the size of the groove-type binding site. The remarkable finding is that very different V_H chains (about 50% homologous) can combine with the same V_K to produce antibody-binding sites with nearly the same size, shape, antigen specificity, and affinity. A similar phenomenon can also occur when different V_H sequences combine with different V_K sequences to produce antibodies with very similar properties. This is a result of the fact that dextran binding depends on the antigen fitting into the groove and interacting favorably with the residues forming the sides and bottom of the groove. The results indicate that divergent variable region sequences, both in and out of the complementarity-determining regions, can be folded to form similar binding site contours, which result in similar immunochemical characteristics. Similar results

V_H19.4.2 and eleven used V_H9.14.7. Starting with
ent V_H genes, these two families of monoclonals
an experiment of nature concerning the ability
V_H gene to combine with different light chain V_K
genes, as well as heavy chain D and J_H genes to pr
groove-shaped binding site of a given specificity.
one of these 21 monoclonals, the same light ch
Ox1 gene was used, but the V_H19 family used a si
sequence exclusively (J_K2), while the V_H9 fam
cluded all four of the active J_K segments (J_K1, 2,
5). Similarly, the heavy chain J_H sequences of th
family were all of a single type (J_H3), while thos
V_H9 family included three types (J_H1, 2, and 3).
D region was used by both families (DFL16.1),
junctional sequences between V_H–D and D–J_H w
ferent, with the V_H19 using minimal substitutio
the V_H9 allowing more variability in junctio
quences, depending on the size of the J_H with v
was joining. Although the amino acid sequences
two V_H genes are 73% identical, they use marke
ferent strategies to arrive at the same groove-typ
ing site with nearly identical size and specifici
results suggest that the two heavy chain variable
perhaps due to their conformation, may place d
structural constraints on which minigene com
can successfully contribute to forming a particu
Two different strategies for generating antibody s
ity are apparent, even though the same V_K and
genes were used by both families. For the V_H19
point mutations in the CDR2 generated the α(
dextran specificity, while the rest of the struct
held constant. For the V_H9 family, a wide variet
J_K, and V_H–D and D–J_H sequences were used t
ate the groove-type site. These two blueprints
structing a binding site may also reflect distinct
pathways for generating antibody diversity.

Protein and Polypeptide Antigenic Determinant

Like the proteins themselves, the antigen
nants of proteins consist of amino acid residues
ticular three-dimensional array. The residues th
contact with complementary residues in the a
combining site are called contact residues. To m
tact, of course, these residues must be exposed
surface of the protein, not buried in the hydr
core. Since the complementarity-determining
in the hypervariable regions of antibodies ha
found to span as much as 30 to 40 Å × 15 to 20
(D. R. Davies, *personal communication*), these

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CHAPTER 8: IMMUNOGENICITY AND ANTIGEN STRUCTURE
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Transforming Growth Factor α: Mutation of Aspartic Acid 47 and Leucine 48 Results in Different Biological Activities

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To study the relationship between the primary structure of transforming growth factor α (TGF-α) and some of its functional properties (competition with epidermal growth factor (EGF) for binding to the EGF receptor and induction of anchorage-independent growth), we introduced single amino acid mutations into the sequence for the fully processed, 50-amino-acid human TGF-α. The wild-type and mutant proteins were expressed in a vector by using a yeast α mating pheromone promoter. Mutations of two amino acids that are conserved in the family of the EGF-like peptides and are located in the carboxy-terminal part of TGF-α resulted in different biological effects. When aspartic acid 47 was mutated to alanine or asparagine, biological activity was retained; in contrast, substitutions of this residue with serine or glutamic acid generated mutants with reduced binding and colony-forming capacities. When leucine 48 was mutated to alanine, a complete loss of binding and colony-forming abilities resulted; mutation of leucine 48 to isoleucine or methionine resulted in very low activities. Our data suggest that these two adjacent conserved amino acids in positions 47 and 48 play different roles in defining the structure and/or biological activity of TGF-α and that the carboxy terminus of TGF-α is involved in interactions with cellular TGF-α receptors. The side chain of leucine 48 appears to be crucial either indirectly in determining the biologically active conformation of TGF-α or directly in the molecular recognition of TGF-α by its receptor.

Transforming growth factor α (TGF-α) is a polypeptide of 50 amino acids. First isolated from a retrovirus-transformed mouse cell line (9), it has subsequently been found in human tumor cells (10, 29), in the early rat embryo (18), and recently in cell cultures from the pituitary gland (23). TGF-α appears to be closely related to epidermal growth factor (EGF) structurally and functionally (19, 20). The two peptides apparently bind to the same receptor, and both induce anchorage-independent growth of certain nontransformed cells, such as NRK cells, in the presence of TGF-β (1).

Comparison of amino acid sequences reveals about 35% homology among the EGF-like peptides (rat [27], mouse [25], and human [13] EGFs and rat [19] and human [12] TGF-αs). Some viral peptides (Shope fibroma growth factor [6], vaccinia growth factor [2], and myxoma growth factor [30]) also share homologies with the EGF-like peptides.

If TGF-α is involved in transformation, a TGF-α antagonist could be an important therapeutic tool in the treatment of certain types of malignancies. An understanding of the conformational and dynamic properties of the TGF-α molecule is basic to the design of an antagonist. A hypothetical antagonist would bind to the same receptor as TGF-α, but would not induce the series of proliferative and transforming events induced by TGF-α. To obtain such a molecule it is necessary to dissociate interactions responsible for binding from those involved in signal transduction. We decided to approach the problem by way of site-directed mutagenesis of a human sequence of TGF-α. In this report we describe our first series of mutations, which were carried out at residues Asp-47 and Leu-48, in the carboxy-terminal part of TGF-α; these two amino acids are highly conserved in the EGF-like family of peptides. We show that these two adjacent residues play different roles in the structure and/or function of TGF-α.

MATERIALS AND METHODS

Cells. Normal rat kidney (NRK) cells were grown in Dulbecco modified Eagle medium containing 10% (vol/vol) calf serum.

TGF-α gene. The sequence of the 50-amino-acid human TGF-α was originally derived from a human TGF-α precursor cDNA (12). The coding sequence is preceded by an ATG methionine codon and followed by a TAA stop codon and is flanked by *Eco*RI restriction sites. This *Eco*RI fragment combines the 59-base-pair *Eco*RI-*Nco*I fragment from plasmid pTE5 (12) with the 111-base-pair *Nco*I-*Eco*RI fragment from plasmid pyTE2 (11). The resulting *Eco*RI fragment was inserted in M13mp18 for site-directed mutagenesis.

Synthesis and purification of oligonucleotides and oligonucleotide-directed mutagenesis. The synthesis and purification of 20- to 27-nucleotide oligonucleotides were carried out as described previously (31). The one or two nucleotides responsible for the mutation were located in the middle of the oligonucleotide. Mutagenesis was performed by published procedures (21, 33). The sequences of the mutant clones were verified by the method of Sanger et al. (25).

Yeast shuttle vector. The vector YEp70αT contains a yeast α-factor pheromone promoter and prepro sequence for the expression of TGF-α (15). The mutant TGF-α coding sequence was inserted in the *Eco*RI site of plasmid YEp70αT and expressed in the form of a fusion protein consisting of 92 amino acids from the prepro sequence of the yeast α factor attached to the amino terminus of TGF-α (28). The yeast cleaves the precursor and secretes TGF-α with 8 amino acids fused to it (4 are encoded by the prepro sequence of α-factor, and the other 4 are encoded by the DNA sequence added to insert of the TGF-α gene). The last of these residues is a methionine, which allows the cleavage of the secreted fusion

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protein by cyanogen bromide (CNBr) and the release of a mature TGF-α (50 amino acids) (see Results).

Yeast strain and transformation. The yeast *Saccharomyces cerevisiae* 20B-12 (*MATα trp1 pep4-3*) (17) was obtained from the Yeast Genetics Stock Center, Berkeley, Calif. *S. cerevisiae* 20B-12 was grown in YEPD medium (1% yeast extract [Difco Laboratories], 2% Bacto-Peptone [Difco], 2% glucose). When the culture reached an optical density at 660 nm of 1, spheroplasts were prepared (14) for transformation. For each transformation we used 10 to 15 μg of purified plasmid DNA.

Partial purification of TGF-α mutants. At 3 days after transformation, five individual colonies of transformants were grown to saturation in YEPD medium. The amount of protein in the yeast medium was measured by the method of Bradford (3), and the amount of mutant TGF-α secreted in the yeast medium was determined by radioimmunoassay. The clones which secrete the highest amount of mutant TGF-α were used to grow a 1-liter culture in YNB-CAA medium (0.67% yeast nitrogen base, 20 g of glucose per liter, 10 g of Casamino Acids [Difco] per liter). After the culture reached saturation (optical density at 660 nm of 10 to 12) (48 h in an air shaker at 30°C), the yeast conditioned medium was dialyzed extensively against 1 M acetic acid in 3,000-molecular-weight cutoff dialysis tubing. Usually 250 ml of dialyzed culture was lyophilized, suspended in 10 ml of 70% formic acid, and treated with CNBr (molar excess of 500) for 20 h at room temperature. The CNBr was subsequently evaporated, and the samples were lyophilized. CNBr-treated samples were suspended in 1 ml of 1 M acetic acid, loaded on a Bio-gel P30 column (30 by 1.5 cm [Bio-Rad Laboratories]), and eluted with 1 M acetic acid. Fractions of 1 ml were collected. Aliquots were lyophilized, suspended in binding buffer (minimum essential medium containing 1 mg of bovine serum albumin per ml and 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.4]), neutralized if necessary to pH 7.4, and tested in EGF-binding competition and soft-agar assays, as well in radioimmunoassay.

Radioimmunoassays. The amounts of TGF-α secreted in the yeast medium were determined by radioimmunoassay with the immunoglobulin G fraction of a polyclonal antibody, 34D, raised against recombinant human TGF-α (4), in 0.1 M Tris (pH 7.5)–0.15 M NaCl–2.5 mg of bovine serum albumin per ml. The amounts of partially purified TGF-α present in the P30 column fractions were measured by using the Biotope RIA kit with polyclonal antibody against human TGF-α (a gift from W. Hargreaves, Biotope), under denaturing conditions, as recommended by the supplier.

EGF binding competition assay and soft agar assay. Both EGF-binding competition and soft-agar assays have been described previously (1).

RESULTS

Rationale for mutations in the carboxyl terminus of TGF-α. Figure 1 shows the amino acid sequence of TGF-α in which the residues that are conserved among all the EGF-like peptides described thus far (EGF, TGF-α, and EGF-like viral proteins) are enclosed in bold circles. Among the 11 conserved amino acids, there are 6 Cys and 2 Gly residues, which presumably play essential roles in determining the overall conformation of the molecule. We concentrated on the two conserved amino acids in the carboxyl terminus, Asp-47 and Leu-48. The Asp in position 47 is conserved among the EGFs and TGF-α (human or murine), but not among the EGF-like viral proteins (vaccinia growth factor, Shope fibroma growth factor, or myxoma growth factor), whereas Leu 48 is conserved among all the EGF-like peptides so far described. In both mouse and human EGF, the two corresponding residues (Asp-46 and Leu-47) are located near the surface of the protein (8, 22, 22a). We designed a series of mutations in these two positions.

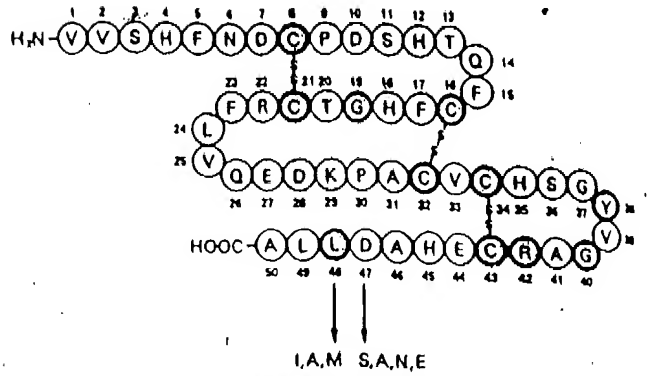

FIG. 1. Mutations in the carboxy terminus of human TGF-α. The amino acids conserved in all the family of EGF-like growth factors (human and murine EGFs and TGFs, as well as the gene products of the vaccinia virus [vaccinia growth factor], the Shope fibroma virus [Shope fibroma growth factor], and the myxoma virus [myxoma growth factor]) are enclosed in bold circles. The mutations of amino acids at positions 47 and 48 are indicated. Symbols: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

Asp-47 has been mutated to Glu, Asn, Ser, and Ala. Glu was chosen because it has the same charge as and a larger size than Asp; Asn has a similar side-chain structure, but is uncharged; Ser is smaller but still polar; Ala is smaller and nonpolar.

Leu 48 has been mutated to Ile and Met, which are both large, nonpolar residues like Leu, and to Ala, which is nonpolar but smaller. We introduced the chosen mutations by site-directed mutagenesis of the cloned human TGF-α gene, using synthetic oligonucleotides.

Construction of the yeast α mating pheromone–human TGF-α plasmid. The TGF-α expression vector pyTE1 (Fig. 2) was constructed by using plasmid YEp70αT (15) which contains the 2μm origin of replication and yeast *TRP1* gene for its replication and selective maintenance, respectively. YEp70αT also contains the yeast α-factor promoter, the α-factor prepro sequence coding for 89 amino acids, and the sequence for 3 amino acids resulting from the introduction of *Xba*I and *Eco*RI sites. The human mature TGF-α sequence (12) is contained in a 170-base-pair *Eco*RI fragment which includes an ATG (Met) codon preceding the sequence of TGF-α and a TAA (stop) codon followed by 8 nucleotides. This TGF-α sequence was inserted in the unique *Eco*RI site of YEp70αT. Clones with the proper orientation were selected, and DNA was isolated for yeast transformation.

Measurement of TGF-α secreted by *S. cerevisiae*. The amount of total proteins secreted into the yeast culture was 10 ± 1 μg/ml for wild-type as well as mutant TGF-α as determined by the method of Bradford (3). Before further purification was attempted, we wanted to determine whether the mutated TGF-α proteins were being secreted by the yeast. The low pH of the yeast medium, as well as the acidic proteins secreted in the yeast culture, precluded biological assay of secreted mutants. Therefore, immunological meth

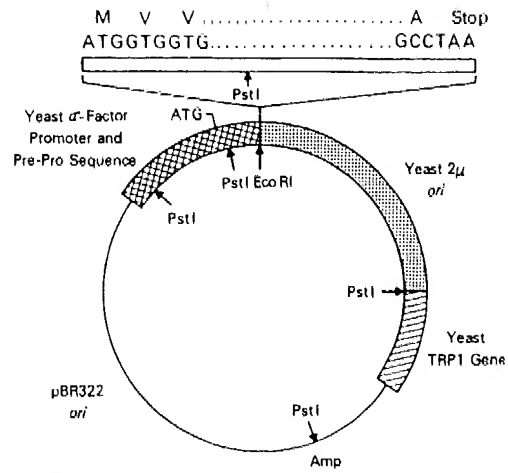

FIG. 2. Structure of the *S. cerevisiae* 8.2-kilobase shuttle vector pyTE1. The secretion of the TGF-α gene is under the transcriptional control of the yeast α-factor promoter and prepro sequence (▨). The yeast 2μm origin of replication (▩) and the selective yeast *TRP1* gene (▧) are indicated. The TGF-α gene, preceded by an initiation (ATG) codon and followed by a stop (TAA) codon, is inserted in the *Eco*RI site. Details are given in Materials and Methods and in Results.

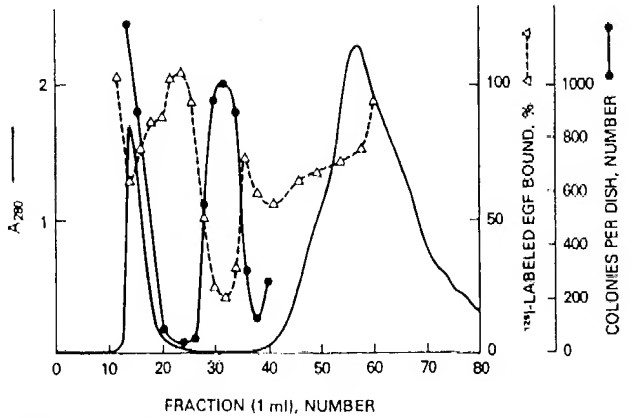

FIG. 3. Purification of yeast-secreted wild-type TGF-α. The purification procedure is described in Materials and Methods and in Results. Aliquots of every other fraction of the Bio-Gel P30 column were tested for their abilities to compete with ^{125}I-EGF for binding to the EGF receptor (△) and to induce colony formation (>62 μm) on NRK cells in soft agar in the presence of TGF-β (1 ng/ml) (●). The A_{280} profile of the proteins was determined (——).

ods were used. Wild-type and mutant TGF-α's were secreted at a level of 100 to 200 ng/ml and 10 to 500 ng/ml, respectively (as determined by radioimmunoassay with polyclonal antibody 34D). We thus estimate that the percentage of TGF-α secreted in the yeast culture is at least 1% of the total protein secreted. We cannot yet assess whether the variations in the levels of secretion of different mutant TGF-α proteins are real or whether one single-amino-acid substitution drastically affects the recognition by the antibody. The latter hypothesis is the more likely, since the use of another polyclonal antibody (Biotope) under denaturing conditions enabled us to detect certain TGF-α mutants (such as [Ala 47]–TGF-α, in which the amino acid in position 47 of human TGF-α is mutated to an alanine) that were poorly detected by 34D, under nondenaturing as well as denaturing conditions. After the amount of TGF-α mutant proteins was estimated, the medium was extensively dialyzed against 1 M acetic acid and lyophilized as described in Materials and Methods.

Partial purification of yeast-secreted TGF-α. Although the yeast shuttle vector was constructed in such a way as to secrete TGF-α with 8 amino acids fused to the N terminus, it was often observed that a significant fraction of the secreted TGF-α was in a higher-molecular-weight fragment corresponding to the size expected from an uncleaved (unprocessed) 92-amino-acid fusion protein. Since a Met had been introduced at the N terminus of TGF-α and since TGF-α contains no Met in its sequence, CNBr treatment could be used to cleave either of these 8- or 92-amino-acid N-terminal peptides and release the complete 50-amino-acid TGF-α. Indeed, CNBr treatment of yeast-secreted proteins resulted in the conversion of high-molecular-weight TGF-α into the 6,000-molecular-weight species, as revealed by Western immunoblot (data not shown).

CNBr-cleaved samples (see Materials and Methods) were purified on a Bio-Gel P30 column. Figure 3 shows the elution profile of the proteins, as well as the results of a radioreceptor assay and a soft-agar assay performed on aliquots of the column fractions. The A_{280} profile shows two major peaks of eluted proteins, one corresponding to the void volume and the other one to proteins of molecular weight <3,000. Aliquots of the column fractions were tested for their ability to compete with ^{125}I-EGF for binding to the receptor. The fractions that were the most active in this assay were located between the two major protein peaks, in an area where relatively few proteins eluted. Although some activity was found in the first protein peak (void volume), this was considerably reduced on treatment with stronger CNBr (data not shown).

Aliquots of each fraction were also tested for their ability to induce anchorage-independent growth of NRK cells in soft agar in the presence of TGF-β (1 ng/ml). The receptor binding and colony-forming activity superimposed almost exactly (Fig. 3). Analysis by polyacrylamide gel electrophoresis with silver staining, as well as by Western blot, of the column fractions shows that our purification procedure (CNBr cleavage followed by P30 sizing column) eliminates high-molecular-weight proteins (data not shown). Since pure TGF-α migrates in a broad band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (32), this technique cannot be used for proper assessment of the degree of separation of TGF-α from low-molecular-weight contaminating proteins. Nevertheless, within our detection levels the amounts of TGF-α present in the column fractions (detected by radioimmunoassay using the antibody from Biotope) correlated with the amounts observed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

Comparison of binding and colony-forming activity of TGF-α partially purified from yeast media. It was important to show that wild-type TGF-α secreted from *S. cerevisiae* had the expected biological properties and that its activity in soft-agar and radioreceptor assays was equivalent. For these assays, the amount of EGF-competing activity present in the most active fraction of the P30 column of wild-type TGF-α was measured in terms of EGF equivalents. The dilution curve had a slope that was parallel to that of the EGF standard. This value was also used to measure the colony-forming activity of the partially purified wild-type TGF-α (with EGF as a standard in the assay). The colony-forming activity of the partially purified wild-type TGF-α corre-

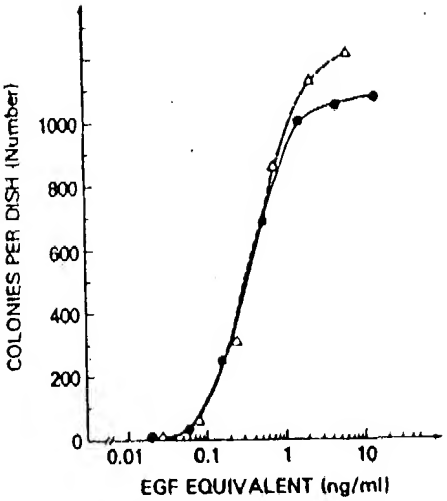

FIG. 4. Correlation between the activities in the binding and colony-forming assay for the partially purified wild-type TGF-α secreted by *S. cerevisiae*. The activity in the radioreceptor assay of the peak fraction from the P30 column was determined in EGF equivalent concentration. The value obtained was used for the soft-agar assay. Colonies of >62 μm (Δ) and the EGF standard (●) are shown.

sponded exactly to that of EGF (Fig. 4). Thus, we have partially purified a wild-type 50-amino-acid TGF-α showing the expected binding and colony-forming activities, which provides a reference substance for mutant TGF-αs that might show a dissociation of binding and colony-forming abilities.

Biological and biochemical activities of the partially purified TGF-α mutant proteins. Mutated TGF-αs were expressed by using the yeast system and partially purified on Bio-Gel P30 columns as described in Materials and Methods. Mutant TGF-αs were usually obtained from two different clones of yeast transformants. The CNBr-cleaved samples were purified through different Bio-Gel P30 columns for each mutant protein to avoid any possible contamination from one peptide to another. The purification profiles observed with the mutant TGF-αs were similar to those obtained for the wild-type TGF-α. Aliquots of the P30 column fractions were tested in radioreceptor and soft-agar assays. For all mutant proteins, the highest activity in both assays was always found in the same fraction of the Bio-Gel P30 column effluent (peak fraction). Extensive purification of a series of mutant proteins for screening purposes is not practical. Therefore, we needed a quantitation system that would allow us to compare mutant proteins with each other. Thus, the amount of TGF-α present in the peak fraction was estimated by radioimmunoassay with an antiserum to native TGF-α (obtained from W. Hargreaves), under denaturing conditions, as described in Materials and Methods. All values given in Table 1 were obtained from the peak fraction.

The controls done with the wild-type TGF-α showed (Fig. 4; Table 1) that binding and transforming activity were equivalent. The yeast vector without a TGF-α insert did not secrete any EGF-like proteins, as determined by both radioreceptor and soft-agar assay.

Two types of results were obtained upon assay of mutant proteins having different amino acid substitutions at Asp-47. In both [Ala-47]–TGF-α and [Asn-47]–TGF-α, binding ability was retained. Soft-agar and radioreceptor activities correlated for [Asn-47]–TGF-α; there was a lower value for

TABLE 1. Biological and biochemical activities of mutant TGF-α proteins secreted by *S. cerevisiae* and partially purified

Insert in the yeast expression vector	EGF equivalence (ng/ml) in:		Amt of TGF-α (ng/ml) in radioimmunoassay
	Radioreceptor assay	Soft-agar assay	
Wild-type TGF-α	700	700	2,000
	400	300	ND[a]
None	0	0	0
[Ala-47]–TGF-α	100	44	220
	66	48	ND
[Asn-47]–TGF-α	80	72	180
	75	72	525
[Glu-47]–TGF-α	3	3	42
[Ser-47]–TGF-α	10	4	60
[Ala-48]–TGF-α	0	0	16
	0	0	220
[Ile-48]–TGF-α	4	12	470
	2	7	490
[Met-48]–TGF-α	2	8	453
	0.5	2	420

[a] ND, Not determined.

colony-forming activity than for EGF-binding competit for [Ala-47]–TGF-α. [Ser-47]–TGF-α and [Glu-47]–TG appeared to have lower activities in both assays than eit wild-type TGF-α or [Ala-47]–TGF-α and [Asn-47]–TGF These results indicate that neither the carboxyl charge the polarity of Asp-47 is essential for biological activity.

The effects of mutation of Leu-48, one of the 11 am acids perfectly conserved among all the EGFs, TGF-αs, viral EGF-like proteins, are dramatic. [Ala-48]–TGF-α tally lacked binding and colony-forming activity. 48]–TGF-α and [Met-48]–TGF-α had very little biolog activity compared with wild-type TGF-α. Another subs tion, [Met-48]–TGF-α, resulted in a truncated mutant lac the last 2 amino acids and having a substitution of Le homoserine at position 48 following treatment with CI Alternatively, if [Met-48]–TGF-α was not treated CNBr, fusion proteins of TGF-α (mutated to Met in pos 48) with 8 or 92 amino acids attached at the N terminus obtained. Very low activities in binding and soft-agar as were found for these mutants, whether or not they cleaved with CNBr. Experiments on EGF and TGF-α shown that an N-terminal extension does not mark modify EGF-binding activity (12, 26). Therefore, the lo activity obtained with [Met-48]–TGF-α that has not CNBr treated was probably due to the mutation itsel not to the N-terminally extended fusion protein. We d know whether the loss of activity observed with the T shortened to 48 amino acids and having a substituti Leu-48 to homoserine is due only to the mutation or a the lack of the last 2 amino acids.

The data obtained by radioimmunoassay on the pa purified wild-type and mutant TGF-α show that the a of TGF-α detected was always higher than the a determined by measurement of biological activity. Thi be due to the presence in the fraction of a certain perc of incorrectly folded TGF-α that might be recognize

radioimmunoassay under denaturing conditions but would not be biologically active. None of the mutant proteins seemed to be present in amounts equivalent to those observed for wild-type TGF-α in the partially purified fractions (whether radioimmunoassay, radioreceptor, or soft-agar assay was used for quantitation). It is not clear whether consistently less TGF-α was produced by the mutant constructs than by the wild type or whether the secreted mutant proteins were simply less well recognized by the antibody. Because of these uncertainties, the biological activities of the different mutant proteins cannot be accurately related to a known amount of mutant TGF-α protein. Even though radioimmunoassay should be used with caution for a quantitative evaluation of mutant TGF-α proteins, a positive reaction demonstrates that immunoreactive TGF-α was present in the P30 peak fraction for each mutant. Therefore, the fact that one of the mutant proteins ([Ala-48]–TGF-α) is biologically inactive can be attributed to the mutation itself, and not to the lack of production of the mutant protein by the yeast or its loss through purification. However, if the mutant proteins are in fact as immunoreactive as the wild type, then [Ala-47]–TGF-α and [Asn-47]–TGF-α are as active as wild-type TGF-α and [Glu-47]–TGF-α and [Ser-47]–TGF-α are less active; in contrast, [Ile-48]–TGF-α and [Met-48]–TGF-α are almost inactive. The differences between mutation of Asp-47 and Leu-48 would then be even more striking.

DISCUSSION

TGF-α shows sequence homologies with EGF, and both growth factors share the same cellular receptors (20). Even though EGF was discovered 25 years ago (7) and its properties have been extensively studied over the years (5), the binding site of EGF to its receptor has still not been determined, and the relationship between structure and function of EGF/TGF-α is still to be discovered. Particularly, we do not know whether binding to the receptor and signal transduction occur through one or more domains of the molecule or through which amino acids. We approached the question by performing site-directed mutagenesis of TGF-α and focused our attention on two adjacent amino acids, Asp-47 and Leu-48, located in the carboxy terminus and highly conserved in the EGF-like family of peptides. Unexpectedly, these two amino acids showed very different sensitivities to mutation and particularly to a substitution to Ala: [Ala-47]–TGF-α retained binding and colony-forming activities, whereas [Ala-48]–TGF-α completely lost both activities. These data show that Asp-47 and Leu-48 play very different roles in defining the structure and/or the activity of TGF-α. The other mutations performed on Asp-47 were substitutions to Asn, Ser, and Glu. [Asn-47]–TGF-α, like [Ala-47]–TGF-α, was active in binding and induction of colony formation, but [Ser-47]–TGF-α and [Glu-47]–TGF-α showed weaker growth factor activities. These results indicate that neither the carboxyl charge nor the polarity of Asp-47 is essential for biological activity. Interestingly, two of the EGF-like viral proteins, myxoma growth factor and Shope fibroma growth factor (6, 30), have Asn instead of Asp in position 47; we have shown that [Asn-47]–TGF-α retains biological activity.

Substitution of Leu-48 to Met and Ile led to mutant proteins with very low activities, whereas substitution to Ala led to complete loss of activity. We did not expect that a mutation of Leu to Ile (which have similar sizes and polarities) would cause such a strong effect. Thus, Leu-48, which is conserved perfectly among all the EGF-like peptides, seems to be essential, through its exact geometry, for the biological activity of TGF-α.

The mutant proteins tested so far, when active, showed parallel behaviors in binding and colony formation. Some mutant proteins lost all activities, and we assume that the binding capacity has been lost. We have not been able to dissociate the binding and colony-forming abilities by using any of the present series of mutant proteins, and it is necessary to screen more of them in search of an antagonist of TGF-α.

Results relating to the biological activity of EGF show that derivatives of mouse EGF and human EGF (EGF 1-47) lacking the carboxy-terminal 6 amino acids as a result of enzymatic digestion are less potent than the intact molecule in mitogenic stimulation of fibroblasts, but retain full biological activity in in vivo assays (inhibition of gastric acid secretion) (16). On the other hand, naturally occurring truncated forms of rat EGF, which lack the carboxy-terminal 5 amino acids (rEGF 2-48) are as potent as mouse EGF (mEGF 1-53) in receptor-binding and mitogenic assays (27). We do not know whether the discrepancies observed are due to the origin of the molecule (artificial or natural) or to the type of bioassay used. In any event, all of these EGF-related molecules, which are shorter than mouse or human EGF, still retain Leu-47. We have shown that in TGF-α, the corresponding residue, Leu-48, is critical for the biological activity.

Recent data on the three-dimensional structure of mouse EGF obtained by nuclear magnetic resonance show that even though Asp-46 and Leu-47 (Asp-47 and Leu-48 in TGF-α) are both solvent accessible (8, 22, 22a), their side chains point in opposite directions in the beta-sheet structure. Therefore, the role of these adjacent amino acids in the structure and, consequently, the function of EGF might be very different. Our data show that the amino acids Asp-47 and Leu-48 of TGF-α are not equally important for the biological activity of TGF-α, despite their conservation among the EGF-like peptides. From the dramatic loss in biological activity which is characteristic of mutation of Leu-48, we also suggest that this residue is involved in binding to the cellular receptors either by direct interaction with the receptor or by providing the proper conformation to the molecule.

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LITERATURE CITED

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Biochemistry

A superactive insulin: [B10-Aspartic acid]insulin(human)

(insulin analogue/peptide synthesis/biological activity)

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ABSTRACT The genetic basis for a case of familial hyperproinsulinemia has been elucidated recently. It involves a single point mutation in the proinsulin gene resulting in the substitution of aspartic acid for histidine-10 of the B chain of insulin. We have synthesized a human insulin analogue, [Asp^{B10}]insulin, corresponding to the mutant proinsulin and evaluated its biological activity. [Asp^{B10}]Insulin displayed a binding affinity to insulin receptors in rat liver plasma membranes that was 534 ± 146% relative to the natural hormone. In lipogenesis assays, the synthetic analogue exhibited a potency that was 435 ± 144% relative to insulin, which is statistically not different from its binding affinity. Reversed-phase HPLC indicated that the synthetic analogue is more apolar than natural insulin. We suggest that the observed properties reflect changes in the conformation of the analogue relative to natural insulin, which result in a stronger interaction with the insulin receptor. Thus, a single substitution of an amino acid residue of human insulin has resulted in a superactive hormone.

Three cases of familial hyperproinsulinemia, a genetic disorder characterized by increased plasma levels of proinsulin-like material, have been recognized (1–3). In two of these cases, the defect has been identified as an amino acid substitution in the proinsulin molecule (4, 5) that resulted in an incomplete cleavage of that molecule. Specifically, cleavage only at the paired basic residues between the B chain and the C peptide occurs (6). In the third case of the disorder, unlike the other two, the proinsulin-like material appeared to be intact proinsulin, suggesting that there was a defect in the prohormone-to-hormone conversion (3). Recent studies have elucidated the genetic basis for the last case of this disorder. Chan *et al.* (7) extracted DNA from leukocytes of the affected family members and, upon cloning, isolated two clones containing the insulin gene. One of these clones contained the normal coding sequence for human preproinsulin, whereas the other clone revealed a single point mutation in the codon for the residue histidine corresponding to position 10 of the B chain of insulin. This mutation, which appears to be implicated as the cause of the hyperproinsulinemia in the affected family (7), predicts a substitution of aspartic acid for this histidine-B10 residue. In view of these findings, it was of interest to synthesize a human insulin analogue that differs from the parent molecule in that the histidine-B10 residue has been replaced with an aspartic acid residue. The present communication describes the synthesis and biological evaluation of this analogue, [Asp^{B10}]insulin(human).

EXPERIMENTAL PROCEDURES

Materials and Analytical Procedures. Details of materials and analytical procedures used in this study have been described (8). The homogeneity of all of the intermediate peptide derivatives was ascertained by thin-layer chromatography on 6060 silica gel (Eastman Chromagram sheet). The solvent systems used were chloroform/methanol/water, 45:10:1, 89:10:1, and 200:75:13 (vol/vol).

Biological Evaluation. The potency of the synthetic analogue was measured in three types of assays: (*i*) insulin receptor binding in a rat liver plasma membrane fraction, in which the relative potency is defined as the ratio of insulin to insulin analogue required to displace 50% of specifically bound ^{125}I-labeled insulin (^{125}I-insulin); (*ii*) lipogenesis in rat adipocytes, in which relative potency is defined as the ratio of insulin to insulin analogue required to achieve 50% of the maximum conversion of [3-^{3}H]glucose into organic solvent-extractable material; and (*iii*) radioimmunoassay, in which insulin or the insulin analogue was used to compete with ^{125}I-insulin in binding to guinea pig antibodies raised against insulin. Complete details of these assays, including sources of reagents and equipment, have been described (9).

Synthesis. [Asp^{B10}]Insulin was prepared by the combination of the S-sulfonated form of human insulin A chain with the synthetic S-sulfonated Asp-10-substituted derivative of human insulin B chain in the presence of dithiothreitol (10). The S-sulfonated human A chain, which is identical with the respective chain of porcine insulin (11), was prepared by oxidative sulfitolysis of porcine insulin and separation of the resulting S-sulfonated A and B chains by column chromatography (12). The synthesis of the S-sulfonated Asp-10-substituted human B chain was patterned after that of the human B chain (13). The C-terminal B-chain hexadecapeptide (residues B15–B30) was coupled with the adjacent hexapeptide (residues B9–B14) to produce the C-terminal docosapeptide (residues B9–B30). This in turn was coupled with the N-terminal octapeptide (residues B1–B8) to yield the protected B-chain analogue which, upon exposure to liquid hydrogen fluoride and oxidative sulfitolysis of the resulting sulfhydryl derivative, afforded the S-sulfonated form of the Asp-10-substituted B chain.

Z-Glu(cHex)-OH·DCHA (Compound I). This compound (where Z is benzyloxycarbonyl, cHex is cyclohexyl, and DCHA is dicyclohexylamine) was prepared from the respective *tert*-butoxycarbonyl (Boc) derivative (Peninsula Laboratories, San Carlos, CA) by deblocking with trifluoroacetic acid and carbobenzoxylation of the ensuing product. The resulting derivative was crystallized from ether as the DCHA salt (mp, 131–132°C). Analysis Calcd for $C_{31}H_{48}N_2O_6$: C, 68.4; H, 8.88; N, 5.4. Found: C, 68.3; H, 9.11; N, 5.1.

Z-Glu(cHex)-Ala-OBu^t (Compound II). Compound I (9.8 g) was partitioned between 0.1 M H_2SO_4 and ethyl acetate, and the organic layer was separated, washed with water, dried ($MgSO_4$), and concentrated to dryness. To a solution of the residue in dimethylformamide (30 ml) cooled to 0°C, alanine *tert*-butyl ester (H-Ala-OBu^t) [prepared from Z-Ala-OBu^t (5.6

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Abbreviations: Bzl, benzyl; Boc, *tert*-butoxycarbonyl; OBu^t, *tert*-butoxy; cHex, cyclohexyl; DCHA, dicyclohexylamine; Ph_2CH, diphenylmethyl; DCC, *N,N'*-dicyclohexylcarbodiimide; HOBT, 1-hydroxybenzotriazole; Z, benzyloxycarbonyl; -ONp, *p*-nitrophenoxy; OBzl, benzyloxy.

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g; OBu^t is *tert*-butoxy) as described (13)] was added, followed by addition of 1-hydroxybenzotriazole (HOBT; 2.3 g) and *N,N'*-dicyclohexylcarbodiimide (DCC; 3.7 g). After 24 hr at room temperature, the urea by-product was filtered off, and the filtrate was diluted with ethyl acetate (500 ml); washed successively with 1 M $NaHCO_3$, water, 0.2 M HCl, and water; dried; and concentrated under reduced pressure to dryness. The product was obtained as an oil [8 g (90%)] that was homogeneous in thin-layer chromatography and was used in the synthesis of the following compound without any further characterization.

Z-Val-Glu(cHex)-Ala-OBu^t (Compound III). Compound II (8 g) in methanol (150 ml) was hydrogenated over a 10% Pd/C catalyst (2 g) for 3 hr. The catalyst was filtered off, and the filtrate was concentrated under reduced pressure to dryness. The residue was mixed with an activation mixture of Z-Val-OH (4.1 g), HOBT (2.7 g), and DCC (3.3 g) in dimethylformamide (30 ml) (activated for 30 min at room temperature before the addition of the amino acid component). After 24 hr the product was isolated in the same manner as described for compound II [oil, 8.7 g (85%)]. This material was homogeneous in thin-layer chromatography and was used in the following synthetic step without further characterization.

Z-Leu-Val-Glu(cHex)-Ala-OBu^t (Compound IV). Compound III (8 g) was hydrogenated as described above, and the resulting oily residue was dissolved in dimethylformamide (30 ml). To this solution benzyloxycarbonylleucine *p*-nitrophenyl ester (Z-Leu-ONp; 5.5 g) and HOBT (1.8 g) were added. After 48 hr, the mixture was diluted with ethyl acetate (250 ml); washed successively with 0.5 M NH_4OH, water, 0.2 M HCl, and water; dried ($MgSO_4$); and concentrated to dryness under reduced pressure. The residue was crystallized from 95% ethanol: weight, 8.4 g (88%); mp, 190–194°C; $[\alpha]_D^{25}$, −20.3° (*c* 1, dimethylformamide). Analysis Calcd for $C_{37}H_{58}N_4O_9$: C, 63.2; H, 8.31; N, 8.0. Found: C, 62.9; H, 8.37; N, 8.1.

Boc-Asp(cHex)-Leu-Val-Glu(cHex)-Ala-OBu^t (Compound V). Compound IV (1.5 g) was hydrogenated as described, and the residue was added to an activation mixture of Boc-Asp(cHex)-OH (Peninsula Laboratories) (0.8 g), HOBT (0.34 g), and DCC (0.52 g) in dimethylformamide (10 ml) (activated for 30 min at room temperature before addition of the amino component). After 24 hr the reaction mixture was processed as described for compound II, and the product was purified by reprecipitation from ethyl acetate/petroleum ether: weight, 1.5 g (85%); mp, 203–205°C; $[\alpha]_D^{25}$ −8.9° (*c* 1, dimethylformamide). Analysis Calcd for $C_{44}H_{75}N_5O_{12}$: C, 61.0; H, 8.72; N, 8.1. Found: C, 60.7; H, 8.56; N, 7.8.

Boc-Ser(Bzl)-Asp(cHex)-Leu-Val-Glu(cHex)-Ala-OH (Compound VI). A solution of compound V (1 g) in trifluoroacetic acid (10 ml) was stored at room temperature for 2 hr and then concentrated to dryness under reduced pressure, and the residue was triturated with cold ether. The solid deblocked pentapeptide trifluoroacetic acid salt formed was filtered and dried over KOH. An activation mixture of Boc-Ser(Bzl)-OH (1.2 g), HOBT (0.5 g), and DCC (0.6 g) in dimethylformamide (10 ml) was prepared; after a 30-min incubation, the mixture was filtered into a solution of the pentapeptide trifluoroacetic acid salt in dimethyl sulfoxide (Me_2SO) (10 ml) containing *N*-methylmorpholine (0.13 ml). After 24 hr the reaction mixture was diluted with cold water (100 ml), and the precipitated product was filtered off, dried, and reprecipitated from ethyl acetate/petroleum ether: weight, 0.9 g (90%); mp, 200–203°C; $[\alpha]_D^{25}$, −17.8° (*c* 1, dimethylformamide). Analysis Calcd for $C_{50}H_{78}N_6O_{14}$: C, 60.8; H, 7.96; N, 8.5. Found: C, 61.4; H, 8.25; N, 8.7. Amino acid ratios after acid hydrolysis: $Asp_{1.0}Ser_{0.8}Glu_{1.0}Ala_{1.0}Val_{1.1}Leu_{1.0}$.

Boc-Ser(Bzl)-Asp(cHex)-Leu-Val-Glu(cHex)-Ala-Leu-Tyr(Bzl)-Leu-Val-Cys(Ph_2CH)-Gly-Glu(Bzl)-Arg(NO_2)-Gly-Phe-Phe-Tyr(Bzl)-Thr-Pro-Lys(Z)-Thr(Bzl)-OBzl (Compound VII). A suspension of the free base of the partially protected hexadecapeptide (residues B15–B30; where Obzl is benzyloxy and Ph_2CH is diphenylmethyl) of the human insulin B chain (8) (400 mg), compound VI (494 mg), and HOBT (80 mg) was stirred until solution occurred. This solution, after the addition of DCC (100 mg), was stirred at 4°C for 48 hr and then diluted with 95% ethanol (150 ml). The precipitated docosapeptide (residues B9–B30) was filtered off, washed with 95% ethanol, and dried: weight, 450 mg (88%). Amino acid analysis after acid hydrolysis gave the following composition expressed in molar ratios: $Asp_{1.1}Thr_{2.0}Ser_{1.0}Glu_{2.1}Pro_{1.0}Gly_{2.3}Ala_{0.9}Val_{1.9}Leu_{2.9}Tyr_{1.9}Phe_{2.0}Lys_{1.1}Arg_{0.7}$ (cysteine was not determined).

H-Phe-Val-Asn-Gln-His-Leu-Cys(SO_3)-Gly-Ser-Asp-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys(SO_3)-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Thr-OH (Human Insulin Asp-10-Substituted S-Sulfonated B Chain) (Compound VIII). A solution of compound VII (400 mg) in trifluoroacetic acid/acetic acid, 7:3 (vol/vol; 10 ml), was stored at room temperature for 1 hr and then diluted with ether. The precipitated trifluoroacetic acid salt of the docosapeptide was filtered off, washed with ether, and dried. A solution of this product in *N*-methylpyrrolidone (6 ml) and dimethylformamide (6 ml) containing triethylamine (0.1 ml) was diluted with ether (100 ml), and the precipitated free base was filtered off, washed with ether, and dried. This product and Boc-Phe-Val-Asn-Gln-His-Leu-Cys(Ph_2CH)-Gly-OH (13) (500 mg) were dissolved in a mixture of dimethylformamide (5 ml) and dimethyl sulfoxide (5 ml) containing HOBT (90 mg), and then DCC (100 mg) was added. After 48 hr at room temperature, the mixture was poured into cold water (250 ml) containing 1 M NH_4OH (5 ml), and the precipitated protected triacontapeptide was filtered off, washed (successively with water, 50% methanol, and methanol), dried, and reprecipitated from dimethylsulfoxide/methanol: weight, 400 mg (90%).

This product was converted to the S-sulfonated Asp-10-substituted B chain by deblocking with liquid hydrogen fluoride, followed by oxidative sulfitolysis as described for the synthesis of S-sulfonated human insulin B chain (13). In a typical experiment, the protected triacontapeptide (200 mg) was treated with anhydrous liquid hydrogen fluoride (9 ml) containing *m*-cresol (1 ml) at 0°C for 1 hr. The hydrogen fluoride was then removed, and the residue was triturated successively with ethyl acetate and petroleum ether. To a solution of this product in 8 M guanidine hydrochloride (20 ml) buffered with 0.1 M Tris·HCl (pH 8.8) were added sodium sulfite (700 mg) and sodium tetrathionate (500 mg). After 3 hr at room temperature, the reaction mixture was placed in Spectrapor membrane tubing no. 3, dialyzed against four changes of distilled water (4 liters each) at 4°C for 24 hr, and lyophilized.

For purification, the lyophilized material was dissolved in 3 ml of 0.04 M acetate/8 M urea, pH 4.0, and applied to a CM-cellulose column (2.5 × 40 cm) that was eluted isocratically with the same buffer. The preparation of column and buffer has been described (12). The column effluent was monitored with an ISCO spectrophotometer (model U-5A), and the elution pattern is shown in Fig. 1. The eluate under the main peak (125–168 ml) was collected and dialyzed as described above; upon lyophilization, the S-sulfonated Asp-10-substituted B chain was obtained as a white powder: weight, 22 mg. Amino acid analysis of the purified chain, after acid hydrolysis, gave the following composition expressed in molar ratios, in agreement with the theoretically expected values: $Asp_{2.1}Thr_{2.1}Ser_{1.1}Pro_{1.1}Glu_{3.0}Gly_{2.9}Ala_{1.0}Val_{3.0}Leu_{3.8}Tyr_{1.8}Phe_{2.9}Lys_{1.1}His_{1.0}Arg_{0.9}$ (cysteine was not determined).

Synthesis and Isolation of [Asp^{B10}]Insulin (Human). To a solution of S-sulfonated porcine (identical with human) A chain (40 mg) and S-sulfonated human Asp-10-substituted B

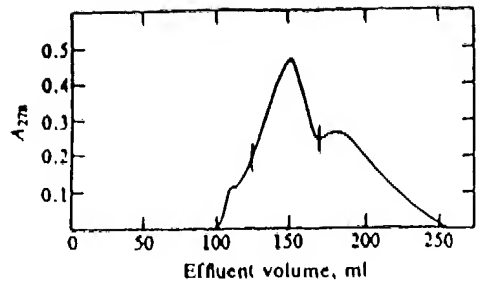

FIG. 1. Chromatography of crude human Asp-10-substituted S-sulfonated B chain on a 2.5 × 40 cm CM-cellulose column with urea/acetate buffer (pH 4.0). The column effluent was monitored with an ISCO recording spectrophotometer. The purified chain (125–168 ml) was recovered after dialysis and lyophilization.

chain (20 mg) in 10 ml of 0.1 M glycine buffer (pH 10.6) cooled to 4°C was added dithiothreitol (7 mg). After 24 hr at 4°C, the mixture was diluted with acetic acid (1 ml), and the resulting precipitate was removed by centrifugation (International HN; 3000 rpm). The supernatant, containing the active material, was passed through a 0.45-μm cellulose acetate filter (Sartorius) and subjected to reversed-phase HPLC using a Vydac 218 TP column (0.45 × 25 cm) connected to an LKB liquid chromatography system. Batches (*ca.* 5 mg of protein each) were chromatographed at a flow rate of 0.5 ml/min with a 10–50% linear gradient of 2-propanol in 0.1% trifluoroacetic acid over 70 min. The chromatographic pattern is shown in Fig. 2*A*. Biological assays indicated that only the material that was eluted at *ca.* 32.3 min had substantial insulin activity. Under these chromatographic conditions, bovine insulin is eluted at 30 min. The fraction containing the active material was concentrated and rechromatographed on the same column with a 20–35% linear gradient of 2-propanol in 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min over 85 min. The elution pattern is shown in Fig. 2*B*. The fraction containing the active material, eluting at *ca.* 47.2 min, was collected and used for biological studies. Under these chromatographic conditions, bovine insulin is eluted at *ca.* 38 min. From the mixture of A and B chains described above, 2 mg of highly purified product was obtained. Amino acid analysis of the purified synthetic material, after acid hydrolysis, gave the following composition, expressed in molar ratios, in good agreement with the theoretically expected values: $Asp_{4.0}Thr_{2.8}Ser_{3.1}Pro_{1.0}Glu_{7.0}Gly_{4.0}Ala_{1.1}Val_{3.4}Ile_{1.4}$-$Leu_{5.9}Tyr_{3.6}Phe_{2.9}Lys_{1.1}His_{1.0}Arg_{1.0}$ (cysteine was not determined).

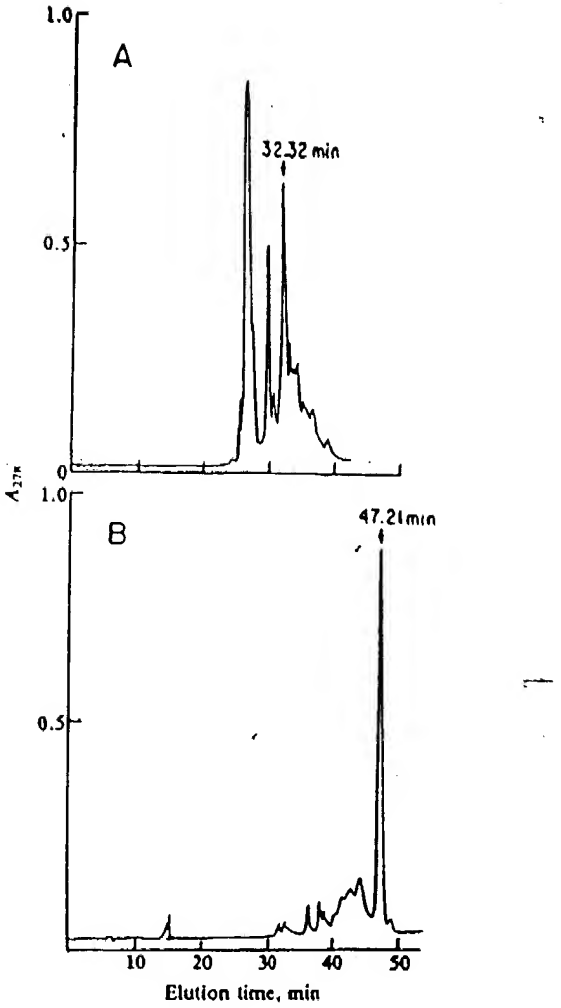

FIG. 2. (*A*) Reversed-phase HPLC of the combination mixture of human S-sulfonated A chain and human Asp-10-substituted S-sulfonated B chain on a 0.45 × 25 cm Vydac 218 TP column at 0.5 ml/min with a 10–50% linear gradient of 2-propanol in 0.1% trifluoroacetic acid over 70 min. (*B*) Rechromatography of the material eluted at 32.3 min in *A*, using the same column and a 20–35% linear gradient of 2-propanol in 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min over 85 min. The active material eluted at 47.2 min was obtained by concentration of the effluent.

RESULTS AND DISCUSSION

The ability of synthetic human [Asp^{B10}]insulin to compete with ^{125}I-insulin in binding to receptors in rat liver plasma membranes was compared with that of natural bovine insulin (Fig. 3). Dose–response curves are essentially parallel, and the calculated potency of the analogue is 534 ± 146% relative to bovine insulin. The stimulation of the incorporation of [3-^{3}H]glucose into organic solvent-extractable material in isolated adipocytes (lipogenesis) was examined for synthetic human [Asp^{B10}]insulin and natural bovine insulin (Fig. 4). The analogue is a full agonist reaching the same maximum stimulation of lipogenesis as seen with natural insulin. However, its relative potency is calculated to be 435 ± 144% relative to bovine insulin. The potency values in receptor binding and lipogenesis assays are not statistically different ($0.4 > P > 0.3$ by Student's *t*-test). It is apparent that [Asp^{B10}]insulin, which differs from human insulin in a single amino acid residue, is a superactive insulin displaying *in vitro* potency 4–5 times greater than that of the natural hormone.† In radioimmunoassay the synthetic analogue exhibited potency approximately equal to that of bovine or porcine insulin. This indicates that substitution of aspartic acid for histidine at position 10 of the B chain has not significantly affected the immunogenic determinants of the molecule.

X-ray analysis indicates that histidine-B10 resides at the surface of the insulin monomer and is important in the formation of zinc insulin hexamers (14). Previous studies in this laboratory showed that replacement of histidine at this position with leucine (15, 16), lysine (17), or asparagine (18) produced synthetic insulin analogues displaying reduced biological activity, *ca.* 14–45% relative to the natural hormone. From these studies we concluded that hydrophilicity

†After the present results were submitted for publication, Jonathan Whittaker (The University of Chicago) determined that [Asp^{B10}]insulin exhibits 5- to 6-fold greater potency than natural insulin in binding to IM-9 lymphocytes (personal communication).

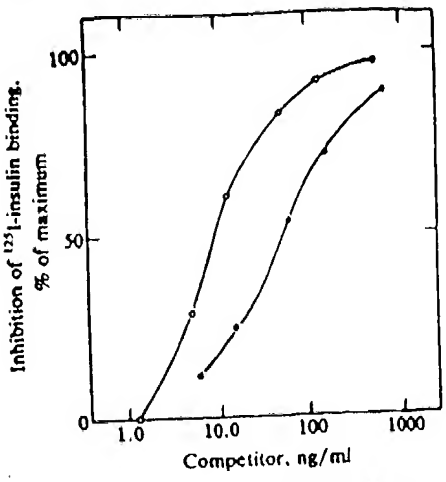

FIG. 3. Effect of bovine insulin (●) and [Asp^{B10}]insulin(human) (○) on the binding of ^{125}I-insulin to rat liver plasma membranes. Inhibition of binding, expressed as percent of maximum, is plotted as a function of the concentration of competitor. The data points represent the mean of triplicate determinations in a representative experiment that was performed four times. In this experiment, the maximum binding amounted to 8.2% of the input radioactivity.

at position B10 *per se* is relatively unimportant in determining biological activity, and the presence of a strongly basic amino acid residue at that position is deleterious. We further suggested that the ability to exist in either a protonated or unprotonated state near physiological pH, a property unique to a histidine residue, might be a requirement at position B10 for high biological activity (17). The present analogue, which at physiological pH would have a negative charge at the B10 position, is several times more active than natural insulin in *in vitro* experiments. The superactivity of this compound results from stronger binding to the insulin receptor. This might be due to either (*i*) a change in conformation of the

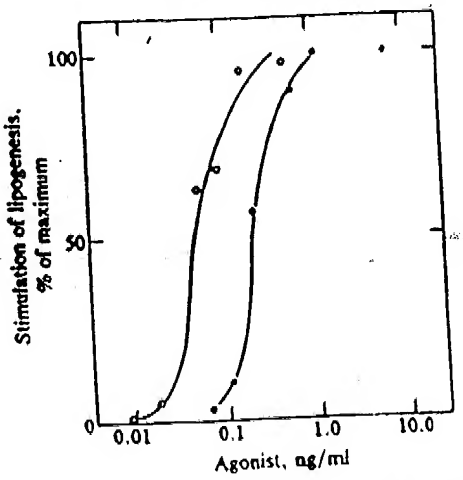

FIG. 4. Effect of bovine insulin (●) and [Asp^{B10}]insulin(human) (○) on the stimulation of lipogenesis in rat adipocytes. Stimulation, expressed as percent of maximum, is plotted as a function of the concentration of agonist. The data points represent the mean of triplicate determinations in a representative experiment that was performed four times. In this experiment, zero and 100% stimulation refer to 0.3 and 3.5 nmol of glucose per mg of cells per hour, respectively.

analogue more favorable for binding to the receptor, resulting from intramolecular interactions involving the negative charge at position B10 (e.g., a salt bridge), or (*ii*) a direct interaction with a complementary surface on the receptor containing a positive charge. In reversed-phase HPLC (Fig. 2), [Asp^{B10}]insulin is eluted under two chromatographic conditions significantly later than is natural insulin. This behavior indicates that the synthetic analogue is a more apolar molecule. The large difference in polarity exhibited between natural insulin and the analogue cannot be ascribed to the substitution of one hydrophilic residue for another. Most reasonably, it reflects a change in conformation. Chan *et al.* (7) have suggested that the inhibition of the conversion of [Asp^{10}]proinsulin to insulin, which results in hyperproinsulinemia in the affected patients, might be related to unfavorable folding of the proinsulin molecule. Since our data on the HPLC behavior of [Asp^{B10}]insulin suggest conformation changes in this molecule as compared to natural insulin, it is not unreasonable to assume that conformation changes might be implicated as well in the abnormal behavior of [Asp^{10}]-proinsulin.

The authors express their appreciation to Dr. D. F. Steiner for communicating to them his early findings concerning [Asp^{10}]proinsulin, to Drs. N. Ferderigos and S. H. Wang for their help in the synthesis of intermediate peptides used in this investigation, and to Dr. U. Roy for the amino acid analyses. This work was supported by the National Institute of Diabetes and Digestive and Kidney Diseases (DK-12925).

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Structure-Function Relationships in Glucagon: Properties of Highly Purified Des-His[1]-, Monoiodo-, and [Des-Asn[28],Thr[29]](homoserine lactone[27])-glucagon†

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ABSTRACT: We have compared the ability of glucagon and three highly purified derivatives of the hormone to activate hepatic adenylate cyclase (an expression of biological activity of the hormone) and to compete with [^{125}I]glucagon for binding to sites specific for glucagon in hepatic plasma membranes. Relative to that of glucagon, biological activity and affinity of [des-Asn[28],Thr[29]](homoserine lactone[27])-glucagon, prepared by CNBr treatment of glucagon, were reduced equally by 40- to 50-fold. By contrast, des-His[1]-glucagon, prepared by an insoluble Edman reagent and highly purified (less than 0.5% contamination with native glucagon), displayed a 15-fold decrease in affinity but a 50-fold decrease in biological activity relative to that of the native hormone. At maximal stimulating concentrations, des-His[1]-glucagon yielded 70% of the activity given by saturating concentrations of glucagon. Thus, des-His[1]-glucagon can be classified as a partial, weak agonist. Highly purified monoiodoglucagon and native glucagon displayed identical biological activity and affinity for the binding sites. Our findings suggest that the hydrophilic residues at the terminus of the carboxy region of glucagon are involved in the process of recognition at the glucagon receptor but do not participate in the sequence of events leading to activation of adenylate cyclase. The amino-terminal histidyl residue in glucagon plays an important but not obligatory role in the expression of hormone action and contributes to a significant extent in the recognition process.

The first event in the cascade of reactions leading to hormone response is the interaction of the hormone with its recognition site termed the "receptor". It is commonly assumed that binding of the hormone to the receptor induces certain transformations in the responding system. Studies of the structural requirements for hormonal recognition and action should provide further understanding of the mechanism of hormone action.

Although the structure of glucagon has been known since 1957 (Bromer et al., 1957), the structure function relationships for this polypeptide hormone have not been elucidated clearly as yet. The discovery of a primary target for glucagon, namely the adenylate cyclase system (for review, see Rodbell, 1972; Sutherland, 1972), has provided a means of evaluating these relationships. Numerous studies have been reported with chemically modified derivatives of glucagon in attempting to establish the structure functional role of each amino acid residue in the peptide (Spiegel and Bitensky, 1969; Rodbell et al., 1971a; Grande et al., 1972; Lande et al., 1972; Epand and Epand, 1972; Epand, 1972; Epand et al., 1973). One major obstacle in obtaining clear-cut results is the purity of the various glucagon derivatives. Chemical modification seldom produces complete conversion of substrate to product. Therefore, extensive purification is essential to remove any remaining native glucagon. Characterization of each derivative requires the study of its affinity for the receptor as well as its intrinsic activity in stimulating adenylate cyclase activity. Obviously, a significant level of contaminating glucagon would make interpretation difficult.

[Des-Asn[28],Thr[29]](homoserine lactone[27])-glucagon (CNBr-glucagon),[1] prepared by cyanogen bromide cleavage of glucagon, has been examined in two studies (Spiegel and Bitensky, 1969; Epand, 1972). In both cases, attempts to separate glucagon from CNBr-glucagon by either gel filtration or gel electrophoresis proved unsuccessful. Unreact-

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[1] Abbreviations used are: CNBr-glucagon, [des-Asn[28],Thr[29]](homoserine lactone[27])-glucagon; DH-glucagon, des-His[1]-glucagon; cyclic AMP, 3′,5′-adenosine monophosphate.

of glucagon was estimated by amino acid analysis to be 2 to 3% of the final product. Thus, while CNBr-glucagon showed reduced activities relative to that of glucagon, it is not known whether this derivative has the same maximal activity as the native hormone. There is also no information available on its affinity for the glucagon receptor.

[^{125}I]Glucagon is commonly used in studies of hormone binding. In the first such study of glucagon binding (Rodbell et al., 1971b), monoiodoglucagon was shown to have an identical concentration-activity dependency as native glucagon on hepatic adenylate cyclase. A recent study (Bromer et al., 1973), however, suggested that monoiodoglucagon has a greater activity in stimulating hepatic adenylate cyclase. If various iodinated derivatives indeed have different potencies from the native hormone, then the interpretation of the binding of [^{125}I]glucagon to membranes containing the glucagon receptor would be difficult.

Des-His^{1}-glucagon (DH-glucagon), prepared by conventional Edman one-step degradation (Rodbell et al., 1971a), showed a lack of biological activity on the hepatic adenylate cyclase system although the derivative binds to specific binding sites for glucagon in hepatic plasma membranes with about one-tenth the affinity of native glucagon. DH-glucagon prepared by Lande et al. (1972), judged by them to contain about 6% glucagon, apparently lacked the ability to activate adenylate cyclase; their studies also indicated that *N*$^{\alpha}$-phenylthiocarbamylglucagon, a potential product of glucagon formed by the Edman procedure, is likely to be biologically inactive.

In this study we have prepared CNBr-glucagon and purified this material to the extent that it contains less than 0.5% native glucagon. DH-Glucagon has been prepared by the insoluble Edman reagent described by Dowling and Stark (1969). In contrast to the conventional method, this procedure allows complete separation from the lysine-substituted derivative. Although the yields of DH-glucagon obtained by this procedure remain to be improved, it is feasible to prepare large quantities containing less than 0.5% glucagon. The glucagon-sensitive hepatic adenylate cyclase system (Pohl et al., 1971) is used for assaying biological activity of monoiodoglucagon, CNBr-glucagon, and DH-glucagon. The affinities of these derivatives for specific glucagon binding sites in hepatic plasma membranes (Rodbell et al., 1971b) have been compared with that of native glucagon as a means of determining the structural requirements for recognition vs. action of the derivatives and the native hormone.

Experimental Section

Materials. [α-^{32}P]ATP was obtained from International Chemical and Nuclear; [^{3}H]cAMP was from New England Nuclear. ATP, cyclic AMP, creatine phosphate, creatine phosphokinase, and dithiothreitol were purchased from Sigma. CNBr was from Eastman Kodak. Oxoid membranes were purchased from Amersham-Searle. Chemicals used in the solid phase Edman procedure were Sequanal grade from Pierce or best grade available and were used without further purification. Polystyrene beads crosslinked with 0.25% divinylbenzene (no. 1.982) (from Sondell Scientific Instruments, Palo Alto, Calif.) were a gift from Dr. George R. Stark. Crystalline porcine glucagon was provided by Eli Lilly and Co. or was purchased from Schwarz/Mann; purified monoiodoglucagon was a gift from Dr. W. W. Bromer of Eli Lilly. Solvents for partition chromatography were purified as previously described (Rodbell et al., 1971).

Preparation of Plasma Membranes from Rat Liver. Partially purified plasma membranes from rat liver were prepared by a modification of the procedure of Neville (1968) as previously described (Pohl et al., 1971), and stored in liquid nitrogen.

Assay for Adenylate Cyclase Activity. The assay medium consisted of [α-^{32}P]ATP (about 40 cpm/pmol) at 2 m*M*; $MgCl_2$, 5 m*M*; cyclic AMP, 0.4 m*M*; dithiothreitol, 1 m*M*; bovine serum albumin, 2 mg/ml; creatine phosphate, 5 m*M*; creatine phosphokinase, 0.3 mg (50 units)/ml; and Tris-Cl buffer, 30 m*M* at pH 7.5 in a final volume of 100 μl. Liver membranes, suspended in Tris-Cl buffer containing 1 m*M* dithiothreitol, were added to a final concentration of 0.2–0.5 mg/ml to initiate the reaction. After 5 min at 30°, the reaction was terminated by adding 100 μl of stopping solution (Salomon et al., 1974). Cyclic AMP formed was determined by a recently developed procedure with the use of Dowex 50 and alumina columns (Salomon et al., 1974).

Assay for [^{125}I]Glucagon Binding. [^{125}I]Glucagon was prepared as described previously (Rodbell et al., 1971b). The concentration of biologically active glucagon in the preparations of labeled hormone was estimated from assays of adenylate cyclase activity (see above); activities were compared with those generated by native glucagon over a wide range of concentrations. The incubation medium used for the binding assay contained [^{125}I]glucagon at 1.5×10^{-9} *M* (see legend for specific activity); bovine serum albumin, 3 mg/ml; liver membrane, 50–100 μg/ml; and Tris-Cl buffer, 20 m*M* at pH 7.5 or as indicated in a final volume of 1 ml. The assay was initiated by the addition of liver membranes and the whole mixture was poured on an oxoid membrane filter after 10 min at 30°. The filter was quickly washed twice with 1 ml of cold Tris-Cl buffer containing 2 mg/ml of bovine serum albumin. Suction was applied during the washing so that each wash took less than 5 sec to complete. Then the filter was counted in a well-type Packard γ-counter.

Analytical Methods. Protein concentration was determined according to Lowry et al. (1951) with serum albumin as standard. The amino acid content was analyzed, after the hydrolysis with methanesulfonic acid (Liu, 1972), on a JEOL JLC-6AM analyzer. No correction is made for destruction of amino acids during hydrolysis. Glucagon concentration was determined by its absorbance at 280 nm (molar absorbance 8050) or by amino acid analysis. Fresh glucagon solution (in 0.1% bovine serum albumin and 20 m*M* Tris-Cl buffer, pH 7.5) was prepared each time prior to use, since we have found that storage of glucagon in solution decreased its effectiveness in activating adenylate cyclase.

Results

Preparation of DH-Glucagon. When DH-glucagon is prepared by the conventional one-step Edman degradation (Rodbell et al., 1971a), the difference between the pK_a values of the α- and ϵ-amino groups is too small to limit the phenyl isothiocyanate reaction to only the α-terminal histidyl residue. Therefore, the product requires extensive purification. It occurred to us that the insoluble Edman reagent (Dowling and Stark, 1969), where products of the reaction of the side-chain ϵ-amino groups are not recovered, might

provide an ideal procedure for the preparation of DH-glucagon. The procedure for preparing glucosaminol-isothiocyanate-polystyrene described by Dowling and Stark (1969) was followed closely. After the reagent was prepared, a column (1 × 15 cm) of the isothiocyanate resin was packed, thoroughly washed with pyridine buffer (pyridine-*N*-ethylmorpholine-H_2O (15:1.4), adjusted to pH 8.1 with glacial acetic acid), and maintained at 55° for the following reactions. Glucagon, 15 μmol, was dissolved in 2.5 ml of the same pyridine buffer and about 5 × 10^6 cpm of [^{125}I]glucagon was added to monitor the recovery. After the sample was placed on the resin, 1 ml of the buffer was added to wash down the sample. All solvents used subsequent to the coupling step contained 1% mercaptoethanol to protect against oxidation. After allowing coupling of glucagon through its amino groups to the resin to proceed for 1.5 hr at 55°, the column was washed twice with 25 ml of pyridine buffer and six times with 25 ml of pyridine, until no radioactivity was detected in the eluate. The resin was washed again with four 50-ml portions of tetrahydrofuran, and the tetrahydrofuran was displaced by adding 3 ml of the cleavage solvent (trifluoroacetic acid-glacial acetic acid (4:1)) to the column; the column flow was stopped and 10 ml of cleavage solvent was added. After 30 min with occasional stirring, this step was repeated once to ensure a good yield of cleavage product. Finally the column was washed with 8 ml of the same solvent. All the cleavage eluates were combined and lyophilized to dryness. The yield of the DH-glucagon at this stage ranged from 10 to 20%. The product obtained at this stage has a brownish color; its amino acid analysis showed the presence of glucosamine and about 0.15 residue of histidine. Since the resin contained covalently bound glucosaminol and after extensive washing the only source for free histidine was the glucagon coupled through ε-NH_2 groups to the resin, it appears that hydrolysis had occurred at the isothiocyanate group and thus glucosaminol and intact glucagon were released. This contaminant was removed by chromatography on a Dowex 50 column. The product was dissolved in 5 ml of 0.1 *M* pyridine-acetate buffer (pH 3.3) and applied to a AG50W-X2 column (0.9 × 9 cm), equilibrated with the same buffer at 55°. After washing the column with 20 ml of 0.77 *M* pyridine-acetate buffer (pH 4.4), DH-glucagon was eluted with 60 ml of 1.38 *M* pyridine-acetate buffer (pH 4.8). After being concentrated to 3 ml, the product was gel filtered on a Sephadex G-10 column (1.5 × 28 cm) with 10% acetic acid and then lyophilized. The yield was 5–10%.

In order to ascertain the amount of unreacted glucagon in the final product, a large amount (20 nmol) of peptide was applied to the amino acid analyzer after methanesulfonic acid hydrolysis. With suitable controls, it was established that the analyzer could detect histidine at levels of 0.1 nmol as a distinct peak. As shown in Table I, DH-glucagon contained less than 0.5% of histidine residue whereas all other amino acid residues, including tryptophan and lysine, were unchanged. We conclude from this analysis that DH-glucagon contains at most 0.5% contaminating glucagon.

Preparation of CNBr-Glucagon. The preparation of CNBr-glucagon followed that general method for cyanogen bromide cleavages of peptides and proteins (Steers et al., 1965). Glucagon (12.0 mg or 3.70 μmol) was dissolved in 12 ml of 70% formic acid (redistilled), and 110 mg of cyanogen bromide was added. The mixture was stirred for 14 hr at 25° and then 30 mg more of cyanogen bromide was added. After a total of 24 hr the solution was lyophilized.

Table I: Amino Acid Compositions of Glucagon and Its Derivatives.[a]

Amino Acids	Porcine Glucagon		DH-Glucagon		CNBr-Glucagon	
	Found	Theory	Found	Theory	Found	Theory
Trp	0.98	1	0.93	1	0.88	1
Lys	1.03	1	1.08	1	1.00	1
His[b]	0.88	1	<0.005	0	0.82	1
Arg	2.00	2	1.83	2	2.00	2
Asp	3.93	4	4.29	4	2.68	3
Thr	2.88	3	2.51	3	1.73	2
Ser	3.46	4	3.38	4	3.60	4
Glu	3.03	3	3.28	3	3.03	3
Gly	0.95	1	1.10	1	1.07	1
Ala	0.93	1	1.03	1	0.93	1
Val	0.89	1	0.90	1	0.86	1
Met[b]	0.93	1	1.07	1	<0.005	0
Leu	2.05	2	2.23	2	2.02	2
Tyr	1.92	2	1.84	2	2.02	2
Phe	2.00	2	1.84	2	2.02	2

[a] Amino acid compositions were analyzed after 20-hr hydrolysis at 115° in 4 *N* methanesulfonic acid containg 0.2% tryptamine. The results expressed in residues per molecule are uncorrected for any destruction during hydrolysis. [b] Histidine and methionine were measured in separate analyses, in which 20 or 30 nmol of DH- or CNBr-glucagon, respectively, was applied. In either analysis, no distinct peak was detected; it was established that the analyzer could detect 0.1 nmol of amino acid as a distinct peak.

A clean separation of native glucagon (R_F 0.32) from CNBr-glucagon (R_F 0.57) was obtained by slightly modifying a solvent system already developed for partition chromatography of glucagon (Hruby and Groginsky, 1971). The system used was 1-butanol-ethanol-benzene-0.2 *N* ammonium hydroxide (5:2:1:8) adjusted to pH 9.4 by acetic acid. The sample was dissolved initially in 1 ml of 50% acetic acid, and then 2 ml of upper phase was added. The mixture was placed on a 2.2 × 57 cm column of Sephandex G-25 (100–200 mesh) which had been equilibrated with the upper and lower phases (Hruby and Groginsky, 1971). The peptide peak was determined using the method of Lowry et al. (1951); the CNBr-glucagon peak was pooled and lyophilized. The peptide was subjected to gel filtration on Sephadex G-15 (1 × 56 cm) using 50% acetic acid. After lyophilization, 3.4 mg of white powder was obtained for a yield of 31%.

Amino acid analysis showed no methionine (<0.5%, Table I) even when the concentration of the analyzed sample was extremely high.

Activation of Hepatic Adenylate Cyclase by Glucagon Derivatives. A typical concentration-activity curve for native glucagon is shown in Figure 1. Activation of adenylate cyclase was detectable at 2 × 10^{-10} *M* glucagon and maximal activity was reached at 4 × 10^{-8} *M* glucagon. By contrast, DH-glucagon did not stimulate adenylate cyclase activity until its concentration reached 3 × 10^{-9} *M*. Furthermore, even at 10^{-6} *M*, a maximal stimulating concentration, DH-glucagon stimulated less than 70% of the maximal activity engendered with native glucagon. Based on the concentration of DH-glucagon required to give activities equivalent to that given with glucagon, it was estimated that the derivative, on a molar basis, has about 2% of the intrinsic activity given by native glucagon. Judging from amino acid analysis (Table I) and the nature of the concentration-ac-

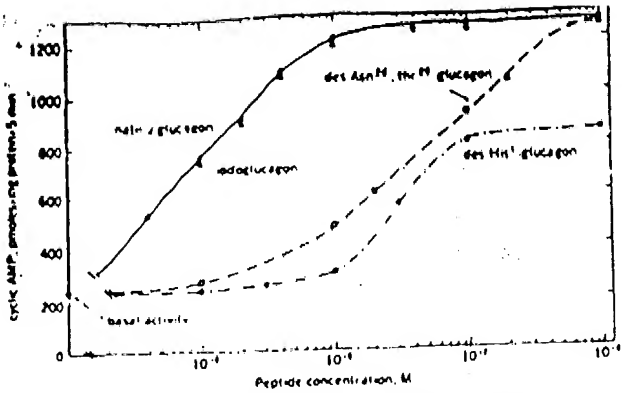

FIGURE 1: Concentration-activity curves for porcine glucagon and its derivatives. The activity of hepatic adenylate cyclase was measured as described in the Experimental Section. The concentration of each peptide was estimated from its absorbance at 280 nm or by amino acid analysis.

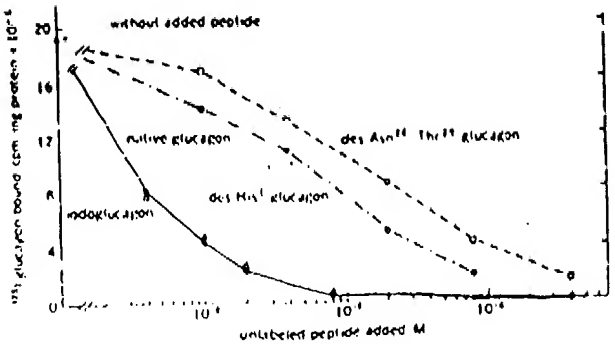

FIGURE 2: Effect of addition of unlabeled peptides on the binding of [^{125}I]glucagon. The binding assays were carried out with 4×10^{-9} *M* [^{125}I]glucagon (specific activity, 10^{6} cpm/pmol) as described in the Experimental Section. Unlabeled peptides were added at the concentrations indicated.

tivity curve, it is highly unlikely that the activity given by DH-glucagon is due to contaminating glucagon. Thus, while confirming a previous report (Rodbell et al., 1971a) that the NH_2-terminal histidine plays an important role in the expression of hormone action, our data show that this residue is not essential for hormone action as previously thought.

In addition to removal of the terminal threonine and asparagine residues of glucagon, CNBr treatment converts methionine to homoserine lactone. These changes in the structure of glucagon reduced its effectiveness to activate the adenylate cyclase system by 40- to 50-fold (Figure 1). However, CNBr-glucagon, in contrast to DH-glucagon, was capable of causing the same degree of activation of adenylate cyclase as the native hormone. Judging from the amino acid analysis (Table I), contaminating glucagon was less than 0.5%; therefore, the activity observed must represent the intrinsic activity of CNBr-glucagon.

It has been reported that the incorporation of iodine into the tyrosyl residues of glucagon reduces the concentration necessary for half-maximal stimulation of adenylate cyclase activity by about fivefold (Bromer et al., 1973). Previously it had been reported (Rodbell et al., 1971b) that glucagon iodinated with I_2 was essentially identical with glucagon in its ability to stimulate adenylate cyclase activity. Because of the widespread use of iodinated glucagon in binding and activity studies, we considered it important to reexamine the effects of monoiodoglucagon, kindly supplied by Dr. Bromer, on the adenylate cyclase system. As shown in Figure 1, native and monoiodoglucagon gave essentially the same concentration-activity curves. As shown below (Figure 2), the relative affinities of glucagon and monoiodoglucagon for the glucagon binding sites in hepatic membranes are also the same.

Glucagon Binding and Competition with Its Derivatives. Hepatic plasma membranes contain binding sites that are highly specific for glucagon (Rodbell et al., 1971b). The relative affinities of native glucagon and the various derivatives for these sites were determined by their ability to compete with the binding of [^{125}I]glucagon to hepatic membranes. As judged by the concentrations required for 50% displacement of the labeled hormone, monoiodoglucagon has the same affinity as native glucagon for the binding sites whereas the affinities of DH-glucagon and CNBr-glucagon for these sites were shifted 15-fold and 50-fold, respectively (Figure 2). It should be emphasized that the loss of affinity of the modified glucagon derivative lacking only histidine was relatively small (15-fold) (in three other experiments of this type, the average was 17-fold) when compared to its loss of ability to activate the adenylate cyclase system (greater than 50-fold). On the other hand, CNBr-glucagon displayed nearly identical decreases in both affinity for the binding sites and in ability to activate the adenylate cyclase system (40- to 50-fold). It is reasonable to conclude from these data that modification at the COOH-terminal region of glucagon by treatment with cyanogen bromide significantly reduces the affinity of the peptide for its receptor but apparently does not affect the intrinsic activity of the hormone derivative as compared to the native hormone. On the other hand, modification of the amino-terminal region of the hormone by removal of the terminal histidyl group not only significantly reduced the affinity of the peptide for these sites, but also decreased substantially the intrinsic activity of the hormone.

Discussion

Our data show that monoiodoglucagon behaves identically with native glucagon in both its binding to specific glucagon binding sites in hepatic plasma membranes and on adenylate cyclase activity in these membranes. Since the preparation of monoiodoglucagon tested in this study was obtained from the same laboratory which reported the increased biological activity of monoiodoglucagon (Bromer et al., 1973), the difference between the two studies is not readily explainable. The tendency of glucagon to aggregate in aqueous solution (Gratzer and Beaven, 1969; Beaven et al., 1969) might be contributory to this discrepancy. We have found that the effectiveness of glucagon on adenylate cyclase activity decreases substantially after storage in solution, especially at concentrations above 10^{-5} *M*. Therefore, we have consistently used freshly prepared solutions of glucagon and the derivatives employed in this study.

It is essential to establish the purity of chemically modified hormones before any meaningful study can be carried out with the derivatives. We have taken great care to make certain that unreacted glucagon remaining after purification was minimal. Careful amino acid analyses indicated that contamination due to native glucagon was less than 0.5% for CNBr- and DH-glucagon. Therefore, the observed properties of these two derivatives cannot be attributed to

the unexpected glucagon. We have shown the [illegible] changes in the properties of glucagon produced by removal of two residues from the COOH-terminal region of glucagon by cyanogen bromide treatment. The contribution of Asn^{28} and Thr^{29} and possibly the terminal carboxy group to the affinity of the hormone is a major one. The participation of these hydrophilic residues (Nozaki and Tanford, 1971) indicates that hydrophobic interactions between glucagon and its receptor are not the only forces involved in the binding process. The parallel loss of both binding and biological activity strongly suggests that the role of the last two residues is strictly in the recognition process. They do not appear to be involved in the sequence of events leading to activation of adenylate cyclase.

It has been reported previously (Rodbell et al., 1971a) that DH-glucagon was devoid of biological activity with only a loss of about tenfold in affinity for the receptor. The implication of this finding and the potential use of this derivative as inhibitor of glucagon action are apparent (Birnbaumer and Pohl, 1973). Our study has confirmed the previous finding that the NH_2-terminal histidine residue in glucagon plays an important role in the expression of hormone action in addition to imparting some contribution to the binding of the hormone to the specific binding sites in hepatic plasma membranes. However, we found that DH-glucagon, prepared and purified as described here, is actually a weak agonist since, at 10^{-6} *M*, the derivative was able to induce about 70% of the maximal stimulation given by 10^{-8} *M* glucagon. Thus, the histidyl residue, though important in the binding and actions of the hormone, does not play an obligatory role in these processes. It is unlikely, therefore, that the histidyl residue of native glucagon participates in a catalytic function as observed for the histidine residue in various enzymes (Moore and Stein, 1973; Bender and Kezdy, 1965). It is possible that the histidyl residue is liganded to an important region of the receptor, or even the catalytic unit of adenylate cyclase, that is important in the expression of hormone action. In this regard, it has been shown that while dissociation of bound glucagon from its receptor is facilitated by the actions of guanine nucleotides on the adenylate cyclase system (Rodbell et al., 1971, 1974), binding of DH-glucagon to the receptor was relatively unaffected (Rubalcava and Rodbell, 1973). Since glucagon and guanine nucleotides activate adenylate cyclase in a concerted fashion (Rodbell et al., 1974), it is conceivable that the histidyl residue of glucagon is involved in this concerted process.

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Possible Dissociation of the Heparin-binding and Mitogenic Activities of Heparin-binding (Acidic Fibroblast) Growth Factor-1 from Its Receptor-binding Activities by Site-directed Mutagenesis of a Single Lysine Residue

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Abstract. The fibroblast or heparin-binding growth factors (HBGFs) are thought to be modulators of cell growth and migration, angiogenesis, wound repair, neurite extension, and mesoderm induction. A better understanding of the structural basis for the different activities of these proteins should facilitate the development of agonists and antagonists of specific HBGF activities and identification of the signal transduction pathways involved in the mechanisms of action of these growth factors. Chemical modification studies of Harper and Lobb (Harper, J. W., and R. R. Lobb. 1988. *Biochemistry.* 27:671–678) implicated lysine 132 in HBGF-1 (acidic fibroblast growth factor) as being important to the heparin-binding, receptor-binding, and mitogenic activities of the protein. We changed lysine 132 to a glutamic acid residue by site-directed mutagenesis of the human cDNA and expressed the mutant protein in *Escherichia coli* to obtain sufficient quantities for functional studies. Replacement of this lysine with glutamic acid reduces the apparent affinity of HBGF-1 for immobilized heparin (elutes at 0.45 M NaCl vs. 1.1 M NaCl for wild-type). Mitogenic assays established two points: (*a*) human recombinant HBGF-1 is highly dependent on the presence of heparin for optimal mitogenic activity, and (*b*) the change of lysine 132 to glutamic acid drastically reduces the specific mitogenic activity of HBGF-1. The poor mitogenic activity of the mutant protein does not appear to be due to a reduced affinity for the HBGF receptor. Similarly, the mutant HBGF-1 can stimulate tyrosine kinase activity and induce protooncogene expression. Differences in the biological properties of the wild-type and mutant proteins were observed in transfection studies. Mutant HBGF-1 expression in transfected NIH 3T3 cells did not induce the same transformed phenotype characteristic of cells expressing wild-type HBGF-1. Together these data indicate that different functional properties of HBGF-1 may be dissociated at the structural level.

THE heparin-binding growth factor (HBGF)[1] family presently consists of seven structurally related polypeptides (3). The cDNAs for each have been cloned and sequenced. Two of the proteins, HBGF-1 and HBGF-2, have been characterized under many different names, but most often as acidic and basic fibroblast growth factor, respectively. Three sequence-related oncogenes have been identified; the hst oncogene was discovered based on its ability to transform NIH 3T3 cells (9, 25, 38, 45); the int-2 oncogene was first identified as a gene activated by mouse mammary tumor virus (7, 10, 11) and the FGF-5 oncogene was identified using NIH 3T3 transformation assays (46, 47). Recently a gene termed FGF-6 was identified by screening a mouse cosmid library with a human hst probe under reduced stringency and was shown to be capable of transforming NIH 3T3 cells (32). Finally, an epithelial cell–specific growth factor termed KGF or FGF-7 has been identified and its cDNA cloned and sequenced (13).

Functions associated with HBGF-1 and HBGF-2 include stimulation of mitogenesis, chemotaxis, mesoderm induction, neurite extension, and plasminogen activator activity. These HBGFs also induce angiogenesis in vivo and accelerate wound repair (for reviews see references 3, 18, 27, 36). The mechanisms by which HBGFs promote these functions are poorly understood but may include activation of protein tyrosine kinase activity (8, 15, 20), phosphorylation of phospholipase C-γ (6), and activation of immediate-early gene transcription (17). In addition, both HBGF-1 and HBGF-2 have been shown to be relatively resistant to degradation after internalization by receptor-mediated endocytosis (14, 24,

1. *Abbreviation used in this paper:* HBGF, heparin-binding growth factor.

34). Intact growth factor persists intracellularly for several hours and large fragments (15,000 and 10,000 M_r for HBGF-1; 16,000 M_r for HBGF-2) are detectable after as many as 24 h. Further, nuclear or nucleolar localization of HBGF-2 has been observed (2, 35).

Despite the identification of additional members of the HBGF family and a broad range of cells and tissues that contain the growth factors, and despite the availability of large quantities of recombinant protein and increased knowledge of the broad spectrum of activities of potential biological significance that can be attributed to the HBGFs, relatively little is known regarding the relationship of these highly conserved structures to any of their known functions. Baird et al. (1) reported the synthesis of 25 peptides, which together encompass and overlap the entire sequence of HBGF-2 as described by Ueno et al. (42). They reported the identification of two functional domains in the primary structure of HBGF-2 based on the abilities of synthetic peptides to interact with HBGF receptor, bind radiolabeled heparin in a solid phase assay, and inhibit HBGF-2 stimulation of thymidine incorporation into DNA. Using the numbering system of the authors (which does not correspond to full length HBGF-2) statistically significant functional activities could be assigned to peptides corresponding to residues 24–68 and 106–115 of HBGF-2. Similarly, Schubert et al. (39) demonstrated that peptides corresponding to residues 1–24, 24–68, and 93–120 of HBGF-2 are able to stimulate substratum adhesion of PC12 cells. We have shown that a synthetic peptide corresponding to residues 49–72 of HBGF-1 (using numbering of 1–154 for full length HBGF-1) is able to compete with HBGF-1 for heparin binding in a gel overlay assay (33). This region is homologous to one of the regions of HBGF-2 (residues 24–68) described above as possessing heparin-binding activity.

To date, the most complete and informative studies documenting the effects of chemical modification of any HBGF on function are those of Harper and Lobb (19). Briefly, they were able to show that limited reductive methylation of bovine HBGF-1 with formaldehyde and cyanoborohydride resulted in stoichiometric methylation only of lysine 132 (using 1–154 numbering for full length HBGF-1). They reported 90% modification of this residue, with 60% dimethylysine. The modified protein exhibited significantly reduced apparent affinity for immobilized heparin (eluted at ~0.7 M NaCl vs. ~1.2 M NaCl for unmodified HBGF-1), a fourfold reduction in its ability to stimulate DNA synthesis in NIH 3T3 fibroblasts and a similar reduction in its ability to compete with labeled ligand in a radioreceptor assay. A lysine residue is found at this position of HBGF-1 and HBGF-2 of all species characterized to date. Together these data implicate a crucial role for lysine 132 in several of the known functions of HBGF-1.

In this report we address the role of lysine 132 in HBGF-1 function using site-directed mutagenesis of this position to a glutamic acid. This approach offers several advantages over chemical modification studies including (*a*) the ability to produce large quantities of the desired product, (*b*) elimination of significant (although sub-stoichiometric) modification of other lysines, and (*c*) allowing the introduction of modified HBGF-1 into mammalian cells through transfection of cDNA expression vectors designed to produce the desired mutant. Despite these advantages the importance of chemical modification studies such as those of Harper and Lobb (19) should not be underestimated for they are extremely useful in the design of a rational approach to site-directed mutagenesis. The results described here demonstrate that replacement of lysine 132 of HBGF-1 with glutamic acid reduces significantly its apparent affinity for immobilized heparin and its mitogenic capacity. However, the apparent affinity of the mutant for high affinity cell surface receptors appears unaltered. When assayed in the presence of heparin where the difference in wild-type and mutant HBGF-1 mitogenic activity is most apparent, mutant HBGF-1 can stimulate tyrosine kinase activity and induce protooncogene expression. Functional differences between the wild-type and mutant HBGF-1 are also apparent after transfection of cDNA expression vectors into NIH 3T3 fibroblasts.

Materials and Methods

Materials

Heparin-Sepharose, protein A-Sepharose, pKK233 expression vectors, and low molecular weight markers were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). All reagents for PAGE and the Mighty Small Apparatus were from Hoefer Scientific Instruments (San Francisco, CA). Reagents for reversed-phase HPLC, amino acid analysis, and amino acid sequencing were purchased from Applied Biosystems, Inc. (Foster City, CA). Isotopes and the in vitro mutagenesis system were from Amersham Corp. (Arlington Heights, IL). The rabbit polyclonal HBGF-1–specific antibody was provided by R. Friesel (American Red Cross, Rockville, MD) and the rabbit polyclonal anti-phospholipase C-γ antibodies were provided by A. Zilberstein (Rorer Biotechnology, Inc., King of Prussia, PA). Tissue culture media and plasticware were purchased from Gibco Laboratories (Grand Island, NY). High molecular weight molecular markers were from Bio-Rad Laboratories (Richmond, CA). Endoproteinase ASP-N and the random primer DNA labeling kit were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Other chemicals were reagent grade.

Construction of pREC and p132E Prokaryotic Expression Plasmids

The plasmid expressing wild-type HBGF-1 (corresponding to the α-form of endothelial cell growth factor (5), pREC, was kindly provided by R. Forough (American Red Cross). This plasmid was constructed by cloning synthetic oligonucleotide cassettes into the Nco I/Hind III site of pKK233-2. The plasmid expressing mutant HBGF-1 (glutamic acid instead of lysine at amino acid position 132; p132E) was constructed as follows. The Eco RI/Hind III fragment of HBGF-1 cDNA clone 1 (21) was subcloned into M13mp18. Single-stranded template was prepared and used for oligonucleotide-directed in vitro mutagenesis. Double-stranded DNA was transformed into *E. coli* TG-1 cells and the resultant plaques were screened by M13 dideoxy sequencing. The mutated HBGF-1 cDNA was transferred into the expression vector pKK223-3 using the original Eco RI and Hind III sites.

Production and Purification of Recombinant Proteins

Recombinant plasmids pREC or p132E were introduced into the *lacI*^{q}-bearing *Escherichia coli* strain JM103. Cultures of JM103 bearing the recombinant plasmids were grown with shaking at 37°C in Luria broth containing 100 μg/ml ampicillin. A fresh overnight culture was diluted and grown until the A_{550} reached ~0.2, at which point isopropylthio-β-galactoside was added to 1 mM. Cells were collected by centrifugation and frozen at −80°C for subsequent growth factor purification.

Thawed cell pellets from 2-liter cultures were resuspended in 50 ml of 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 50 mM glucose. A fresh solution of hen egg lysozyme in the same buffer was added to 10 μg/ml. The cells were mixed at 4°C for 45 min. The viscous lysate was sonicated at maximum intensity using a large probe and four 20-s pulses of a Heat Systems W-380 sonicator. The lysate was clarified by centrifugation at 6,000 *g* for 15 min at 4°C. The supernatant was diluted to 100 ml with 50 mM Tris-HCl, pH 7.5, 10 mM EDTA and incubated with 20 ml of hydrated heparin-

Sepharose at 4°C with end-over-end mixing for 2 h. The resin was eluted batchwise using a sintered glass funnel and successive washes of the same buffer containing 0, 0.1, 0.5, 0.65, and 1.5 M NaCl.

The wild-type recombinant HBGF-1 eluted with the 1.5 M NaCl wash. The mutant was eluted with the 0.5 M NaCl wash. Although the wild-type protein was essentially pure after heparin-Sepharose chromatography, the mutant HBGF-1 constituted only 10–20% of the 0.5 M NaCl wash. Both preparations were purified to >95% purity using reversed-phase HPLC (4). The reversed-phase purified material was used for all reported studies.

Characterization of Recombinant Proteins

All preparations of purified recombinant human wild-type and mutant HBGF-1 were analyzed by SDS-PAGE, amino acid analysis, amino terminal sequencing, peptide mapping, and amino acid sequencing of the peptide encompassing the mutated residue. Protein concentrations were determined by amino acid analysis. Aliquots of wild-type and mutant HBGF-1 were subjected to electrophoresis using the SDS-PAGE system of Laemmli (26). A 15% acrylamide, 0.4% *N,N'*-methylenebisacrylamide solution was polymerized in a Hoefer mini-gel apparatus and electrophoresis was carried out at a constant 200 V. Protein was visualized by staining the gel with 0.1% Coomassie blue R-250 in 50% methanol, 10% glacial acetic acid, and destaining with 9% glacial acetic acid, 5% methanol. Samples for amino acid analysis were hydrolyzed with argon-purged, constant boiling 6 N HCl at 115°C for 18 h using a Pico-Tag workstation (Waters Associates, Milford, MA). Amino acids were derivatized with phenylisothiocyanate and separated with a PTC analyzer (model 130A; Applied Biosystems, Inc.). A Waters 840 system was used for data collection and reduction. Amino acid sequences were established using a protein sequencer (model 477A; Applied Biosystems, Inc.) using modified Edman chemistry and an on-line model 120A PTH analyzer. Peptide mapping of recombinant protein after digestion with endoproteinase Asp-N at a 1:25 ratio of enzyme to protein in 50 mM Na_2HPO_4, pH 8.0, 37°C for 18 h was performed using a microbore HPLC system (model 130A; Applied Biosystems, Inc.). The appropriate peptides were subjected to amino acid sequence analysis to establish the fidelity of expression of the wild-type and mutant HBGF-1 vectors.

Stability Studies

Metabolically labeled recombinant proteins were prepared by growing bacterial cultures as described above until the A_{550} reached ~0.4, at which point the cells were collected by centrifugation. They were resuspended in 98.5% M9 minimal medium/1.5% Luria broth and [^{3}H]leucine (140 Ci/mmol) was added to 45 μCi/ml. Cells were grown with shaking for 30 min, and then for an additional 4 h in the presence of 1 mM isopropylthio-β-galactoside. Cells were collected and growth factors purified as described above. The purified, labeled growth factors were incubated for 48 h at 37°C in the presence of media (DMEM containing 10% calf serum) that had been conditioned for 48 h by NIH 3T3 cells. The growth factor–containing media was analyzed by SDS-PAGE and autoradiography.

Mitogenic Assays

The mitogenic activities of wild-type and mutant recombinant HBGF-1 were determined by measuring their ability to stimulate DNA synthesis in NIH 3T3 cells and to support the proliferation of human umbilical vein endothelial cells. DNA synthesis was determined by measuring the amount of [^{3}H]thymidine incorporated into cells. Briefly, NIH 3T3 cells were seeded into 48-well plates and grown to near confluence in DME containing 10% calf serum. The cells were serum starved (DME, 0.5% calf serum) for 24 h. Mitogens were added to the wells and incubated for 18 h. The cells were pulsed with 0.5 μCi/ml of [^{3}H]thymidine (25 Ci/mmol) for 4 h. The cells were rinsed with PBS, fixed with 10% TCA, rinsed with PBS, and then solubilized with 0.5 N NaOH. Incorporation of [^{3}H]thymidine into acid-insoluble material was determined by scintillation counting. All assays were performed in triplicate.

Human umbilical vein endothelial cells were provided by T. Maciag (American Red Cross, Rockville, MD). They were maintained on fibronectin-coated plates (2 μg/cm^2) in medium 199 supplemented with 10% (vol/vol) heat-inactivated FBS, 1× antibiotic–antimycotic, 10 U/ml heparin, and 10 ng/ml human recombinant HBGF-1. For growth assays, cells were seeded in 24-well plates at 2,000 cells/well in medium 199 supplemented as above with the exception of HBGF-1. The indicated amounts of wild-type or mutant HBGF-1 and heparin were added to the wells. The media was changed ever other day. After 7 d in culture, cells were trypsinized and counted using a hemocytometer.

Competition for Binding and Cross-Linking to Cell Surface Receptors

Bovine brain–derived HBGF-1 (4) was labeled with ^{125}I using immobilized lactoperoxidase and biologically active, labeled protein was isolated using heparin-Sepharose as described (16). Confluent NIH 3T3 cells in 24-well plates were serum starved for 24 h before binding experiments in DME containing 0.5% calf serum. The cells were washed and incubated with DME containing 5 U/ml heparin, 0.5% BSA, and 25 mM Hepes, pH 7.2 (binding buffer) at room temperature for 20 min. The cells then were incubated with ^{125}I-HBGF-1 and unlabeled wild-type or mutant HBGF-1 in the presence of 5 U/ml heparin as indicated in the figure legend. The cells were incubated on ice for 90 min. The plates were aspirated and washed four times with binding buffer. The cells were then incubated for 20 min at 4°C with 1 ml of 0.3 mM disuccinimidyl suberate in PBS. The cross-linker was then aspirated off and the reaction quenched by adding 2.0 M Tris-HCl, pH 8.0. The cells were washed with PBS, scraped from the plates and pelleted for 10 s at 15,000 *g*. The pellets were extracted with 100 μl of 50 mM Tris, 1 mM EDTA, 200 mM NaCl, 1.0% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.5 for 20 min at 4°C. The extracts were centrifuged for 10 min at 15,000 *g*. The supernatants were removed and mixed with an equal volume of Laemmli sample buffer for SDS-PAGE analysis.

Stimulation of Protein Tyrosine Kinase Activity

NIH 3T3 cells were grown to confluence in 100 mm dishes and serum starved as described above. The cells were then exposed to diluent 1.0, or 10 ng/ml of wild-type or mutant HBGF-1 for 10 min at 37°C. The cells were washed once with cold PBS then lysed in buffer containing 10 mM Tris, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1.0% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, pH 7.4. The cells were scraped from the plates, vortexed, and incubated on ice for 10 min. Lysates were clarified by centrifugation at 10,000 *g* for 10 min at 4°C and the supernatants were mixed with an equal volume of 2× Laemmli sample buffer. Samples (normalized to cell number) were subjected to PAGE in the presence of SDS. The proteins were transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine antibodies as described (15). The blots were incubated with ^{125}I-protein A and phosphotyrosine-containing proteins were visualized by autoradiography. In some experiments the initial cell lysates were incubated with a pre-bound anti–phospholipase C-γ antibody/protein A–Sepharose complex (31) for 90 min at 4°C. The beads were washed with 20 mM Hepes, 0.1% Triton X-100, 150 mM NaCl, 10% glycerol, pH 7.5. Immunoprecipitated proteins were eluted from the beads with 2× Laemmli sample buffer and subjected to PAGE and Western blotting with anti-phosphotyrosine antibodies as described above.

RNA Gel Blot Analysis

NIH 3T3 cells were incubated for 48 h in DME/0.5% FCS and then either left unstimulated or stimulated with wild-type or mutant HBGF-1 for the indicated times. Cells were harvested, total RNA was prepared (17), and 10 μg of each sample was separated by electrophoresis on 1.2% agarose gels containing formaldehyde. The gels were stained with ethidium bromide photographed to verify that each lane contained an equal amount of undegraded ribosomal RNA. RNA was electroblotted onto Zetabind nylon filters and cross-linked by UV irradiation. The restriction fragments used and source of the DNA probes were as follows: (*a*) c-*fos*, 2.8-kb Nco I/Xho I fragment of pc-*fos*-1; American Type Culture Collection, Rockville, MD; (*b*) c-*jun*, 1.5-kb Hind III/Bam HI fragment of ph-cJ-1; gift of P. Angel, University of California, La Jolla, CA; (*c*) c-*myc*, 1.4-kb Sst I fragment of pHSR-1; ATCC; (*d*) glyceraldehyde 3-phosphate dehydrogenase, 0.8-kb Pst I/Xba I fragment of pHcGAP; ATCC. The probes were labeled with [^{32}P]dCTP (3,000 Ci/mmol) using a random primer labeling kit. Hybridization and filter washes were as described (17). Blots were exposed to Kodak XAR5 film at −70°C.

Transfection of NIH 3T3 Cells with HBGF-1 Eukaryotic Expression Plasmids

NIH 3T3 cells in 100 mm dishes were transfected with plasmid DNA by the calcium phosphate precipitation method (44). Cells were incubated with either 1 μg of pSV2 neo (41) or co-transfected with a mixture (1:10 μg) of pSV2 neo and either HBGF-1 wild-type expression vector (p267) or HBGF-1 mutant expression vector (p268). The plasmid p267 is described in Jaye et

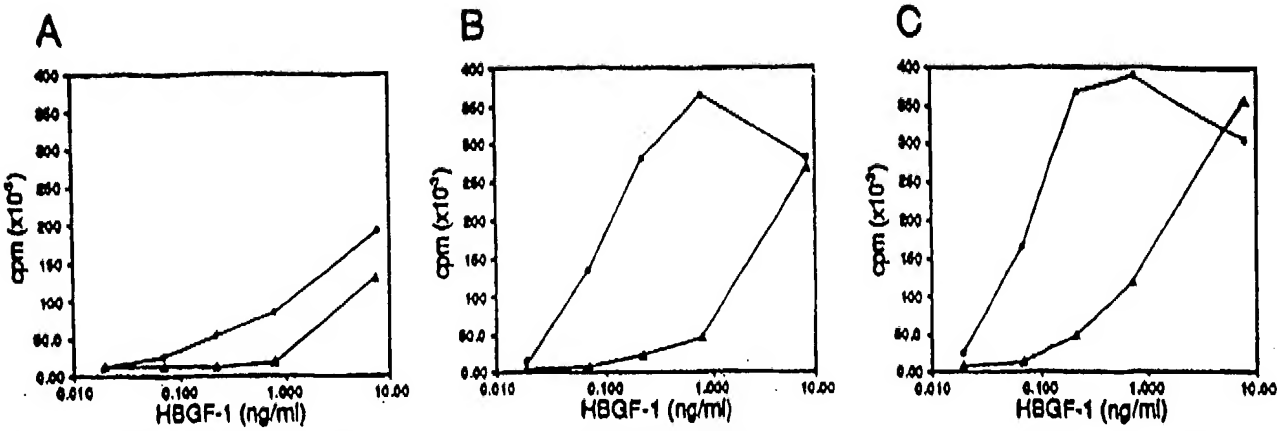

Figure 1. Stimulation of DNA synthesis in NIH 3T3 cells by wild-type and mutant HBGF-1. Cells were grown to near confluence and serum starved for 24 h as described in Materials and Methods. Cells were treated with the indicated concentrations of wild-type (●) or mutant (▲) HBGF-1, incubated for 18 h, and then pulsed with 0.5 μCi of [³H]thymidine/ml for 4 h. The cells were harvested and incorporation of radioactivity was determined. Both wild-type and mutant HBGF-1 were assayed in the presence of 0 (*A*), 5 (*B*), or 50 U/ml heparin (*C*).

al. (23); p268 was constructed by replacing the 297nt Pvu II/Bgl II fragment of p267 (encoding amino acids 38–155) with the corresponding region from the prokaryotic expression plasmid pE132 using standard subcloning methods. Cells were split to 10 dishes and transfected colonies were selected by incubating the cells in DME, 10% calf serum containing 500 μg/ml Geneticin. The media was changed every 3–4 d. After 4 wk, transfected colonies were analyzed for HBGF-1 expression by Western blot analysis using rabbit polyclonal HBGF-1–specific antibodies and ^{125}I-protein A as described above.

Results

Heparin-binding Properties of HBGF-1 Mutant p132E

A drastic reduction in the apparent affinity of HBGF-1 containing glutamic acid in place of lysine at position 132 was observed during the purification of the recombinant proteins from the *Escherichia coli* lysates. Recombinant wild-type HBGF-1 from *E. coli* lysates can be purified to near homogeneity with a single heparin-Sepharose step. The protein binds the immobilized heparin during extensive washing with 0.5 and 0.65 M NaCl–containing buffers and is eluted with a single step of 1.5 M NaCl–containing buffer. In contrast, heparin-Sepharose affinity-based chromatography could not be used as a single purification step for the mutant HBGF-1. The mutant protein binds immobilized heparin in the presence of 0.1 M NaCl but was eluted during the 0.5 M NaCl wash. Both wild-type and mutant HBGF-1 (1.5 and 0.5 M NaCl eluates, respectively) could be purified to apparent homogeneity using reversed-phase HPLC. Detailed analysis of the apparent affinities of the two purified proteins for immobilized heparin-Sepharose using relatively shallow, linear NaCl gradients indicated that the mutant HBGF-1 eluted with 0.45 M NaCl whereas wild-type required 1.1 M NaCl to be eluted (data not shown). For all of the assays described below we used reversed-phase HPLC purified wild-type or mutant HBGF-1. Protein concentrations were determined by amino acid analysis of preparations that had been shown to be the desired HBGF-1 form by peptide mapping and amino acid sequence analysis (data not shown).

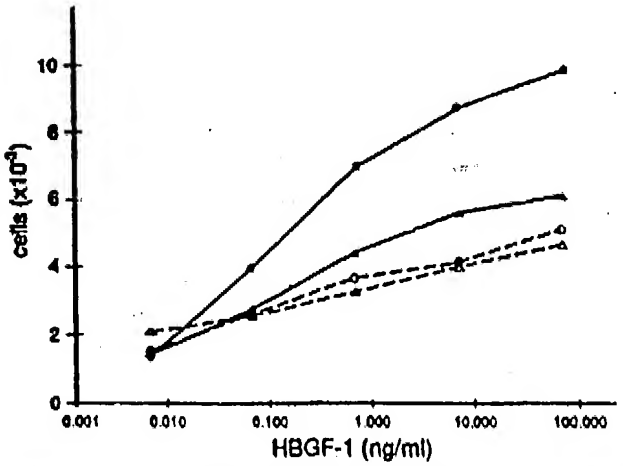

Figure 2. Ability of wild-type and mutant HBGF-1 to stimulate growth of human umbilical vein endothelial cells. Cells were seeded and cultured as described in Materials and Methods. Cell number after 7 d in culture in the presence of the indicated concentrations of wild-type (○/●) or mutant (△/▲) HBGF-1 in the absence (○/△) or presence (●/▲) of 50 U/ml heparin is shown.

Mitogenic Properties of HBGF-1 Mutant p132E

The ability of the HBGF-1 mutant to stimulate mitogenesis was compared to that of the wild-type protein using two different assays. In the first, the ability of the two proteins to stimulate DNA synthesis in NIH 3T3 cells as measured by [³H]thymidine incorporation was examined. The assays were conducted over a broad range of HBGF-1 and heparin concentrations. Two important points can be made from the data in Fig. 1. One, the wild-type HBGF-1 has a dramatic requirement for the presence of heparin for optimal mitogenic activity and, two, the mutant HBGF-1 is significantly less potent than wild-type protein in the presence of added heparin. As can be seen in Fig. 1, the maximal difference in mitogenic potency was observed in the presence of 5 U/ml heparin (~30-fold). Little difference (approximately three-

Table I. Cell Number (× 10^{-5})

	Growth factor concentration (ng/ml)					
	0	0.1	0.5	1	5	10
GLU_{132} HBGF-1	1.6	1.6	1.3	1.2	1.7	1.4
Wild-type HBGF-1	1.7	2.0	1.9	2.9	12.6	16.6

fold) between the wild-type and mutant protein is seen in the absence of added heparin because of the relative lack of mitogenic activity of wild-type human recombinant HBGF-1 in the absence of heparin. The possibility that the reduced mitogenic activity of the mutant HBGF-1 is related directly to its reduced apparent affinity for immobilized heparin is supported by the observation that the difference in the mitogenic potency between the wild-type and mutant protein is reduced to ~18-fold in the presence of 50 U/ml heparin.

In the second mitogenesis assay the abilities of the wild-type and mutant proteins to support the proliferation of human umbilical vein endothelial cells were compared. The results shown in Fig. 2 are consistent with those described above in that they demonstrate a dramatic heparin requirement of the wild-type HBGF-1 for biological activity and that the mutant HBGF-1 is not able to support cell proliferation to the same extent as the wild-type protein. These experiments were conducted in the presence of 50 U/ml heparin and the endothelial cells were seeded in the presence of 10 ng/ml wild-type HBGF-1. When growth assays were conducted in the presence of 5 U/ml heparin without wild-type protein during the seeding, mitogenic deficiencies of the mutant protein were more pronounced (Table I). The results shown in Fig. 3 demonstrate that the reduced mitogenic activity of the mutant HBGF-1 does not appear to be the result of any increased susceptibility of the protein to proteolytic digestion by components in serum or the conditioned media of NIH 3T3 cells.

Receptor-binding Activity of HBGF-1 Mutant pI32E

The results presented above are consistent with the observations of Harper and Lobb (19) using bovine brain-derived HBGF-1 selectively methylated at lysine 132, although the magnitude of the reduction in mitogenic potency (~30-fold for 3T3 cell assay) as compared with the ~4-fold decrease reported by Harper and Lobb (19) is significantly greater. They also reported reduced receptor-binding activity for the modified protein. We examined the abilities of the wild-type and mutant recombinant HBGF-1 to compete with ^{125}I-labeled bovine HBGF-1 for binding to cell surface receptors on NIH 3T3 cells at a concentration of added heparin (5 U/ml) where the difference in mitogenic potencies of the two proteins was greatest.

The receptor-binding activity of the mutant HBGF-1 was established by competition for cross-linking of ^{125}I-HBGF-1 to 150,000- and 130,000-M_r proteins present on the surface of NIH 3T3 cells (16). The results shown in Fig. 4 demonstrate that the mutant HBGF-1 is similar to wild-type protein in its ability to compete for receptor-ligand cross-linking.

The functional consequences of HBGF-1 binding to its cell surface receptor include stimulation of protein tyrosine kinase activity (8, 15, 20) including phosphorylation of phospholipase C-γ (6). Fig. 5 *A* demonstrates that both wild-type and mutant HBGF-1 are able to increase the phosphotyrosine content of 150,000-, 90,000-, and 70,000-M_r proteins and, to a lesser extent, proteins with lower relative molecular masses as judged by Western blot analysis with phosphotyrosine-specific antibodies. The dose response and extent of activation is similar for the two forms of the growth factor. Stimulation of the phosphotyrosine content of phospholipase C-γ was examined by anti-phosphotyrosine Western blot analysis of 3T3 cell lysates after immunoprecipitation using antibodies that recognize phospholipase C-γ. Fig. 5 *B* demonstrates that mutant HBGF-1 shares with wild-type HBGF-1 the ability to stimulate tyrosine phosphorylation of phospholipase C-γ. These data regarding stimulation of tyrosine kinase activity by wild-type and mutant HBGF-1 are in good agreement with the receptor-binding data described above but do not provide insight into the functional basis for the relatively poor mitogenic capacity of this HBGF-1 mutant.

Protooncogene Induction by Wild-Type and Mutant HBGF-1

The results described above indicate that the functional properties of the mutant HBGF-1 associated with events that occur at the cell surface (i.e., receptor-binding and tyrosine kinase activation) are normal with respect to those of wild-type HBGF-1. In addition to tyrosine kinase activation, another early response to HBGF-1 receptor-binding is the elevation of protooncogene mRNA levels (17). To determine the effect of wild-type and mutant HBGF-1 on protooncogene expression, NIH 3T3 cells were serum starved and then either left unstimulated or stimulated with 10 ng/ml wild-type or mutant HBGF-1. Heparin (5 U/ml) was also added to the cells receiving growth factor. Cells were collected at various times after stimulation, RNA was prepared, and levels of c-*fos*, c-*jun*, c-*myc*, and glyceraldehyde 3-phosphate dehydrogenase mRNA (as a control for the amount of RNA loaded in each lane) were assayed by RNA gel blot analysis. Wild-type and mutant HBGF-1 increased protooncogene mRNA levels to a similar degree; maximal levels were observed at 30 min (c-*fos*, c-*jun*) or 2 h (c-*myc*) after stimulation (Fig.

Figure 3. Analysis of the relative stability of wild-type and mutant HBGF-1 in NIH 3T3 cell-conditioned media. The wild-type and mutant proteins were labeled and purified as described in Materials and Methods. The proteins were incubated in the presence of NIH 3T3 cell-conditioned media for 48 h at 37°C and then subjected to SDS-PAGE. The gels were dried and labeled proteins visualized by autoradiography. Lane 1 contains wild-type HBGF-1 and lane 2 mutant HBGF-1. The apparent molecular weights of both proteins are identical to that of HBGF-1 before incubation.

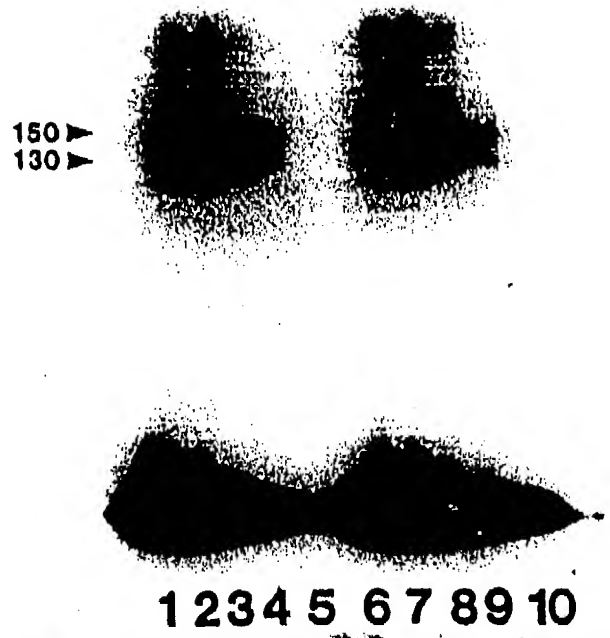

Figure 4. Ability of wild-type and mutant HBGF-1 to compete with ^{125}I-labeled bovine HBGF-1 for cross-linking to 150,000- and 130,000-mol wt cell surface receptors. NIH 3T3 cells were incubated with 1 ng/ml bovine ^{125}I-HBGF-1 and either 0.5, 1.0, 5.0, 10.0, or 50.0 ng/ml of wild-type (lanes 1–5) or mutant (lanes 6–10) human recombinant HBGF-1 in the presence of 5 U/ml heparin. After incubation, the cells were treated with cross-linking reagents as described in Materials and Methods. The apparent molecular weights of cross-linked species were determined after SDS-PAGE and autoradiography. The positions of two cross-linked 150,000– and 130,000–mol wt species, which correspond to the known apparent molecular weights of HBGF receptors, are indicated with arrows.

6). The addition of heparin alone did not induce protooncogene expression. Since the mitogenic differences between the wild-type and mutant HBGF-1 are more pronounced at lower growth factor concentrations, we also stimulated cells with 0.5, 1.0, 5.0, and 10 ng/ml wild-type and mutant growth factor (again in the presence of heparin). At all four concentrations used, the wild-type and mutant HBGF-1 were similar in their ability to induce c-*fos* mRNA expression (Fig. 7).

Overexpression of Wild-Type and Mutant HBGF-1 in Transfected NIH 3T3 Cells

It was demonstrated previously that overexpression of wild-type HBGF-1 in transfected Swiss 3T3 cells resulted in cells with an elongated, transformed morphological phenotype that grew to higher saturation densities (23). This transformed phenotype occurred even though the HBGF-1 polypeptide was not detectable in the conditioned media of these cells. We have shown that the mutant HBGF-1 is not a potent mitogen although it can bind receptor and initiate early events associated with mitogenic signal transduction. To investigate whether the intracellular function of the mutant HBGF-1 was altered, we examined the ability of this protein to induce a transformed phenotype in NIH 3T3 cells. Cells were either transfected with a plasmid conferring neomycin resistance or co-transfected with the neomycin resistance plasmid and wild-type or mutant HBGF-1 expression vectors.

Fig. 8 shows the results of Western blot analysis of transfected cell lysates using HBGF-1–specific antibodies. The Western blot analysis was normalized to cell number and provides the basis for our designation of relatively high or low levels of HBGF-1 expression. The results shown in Fig. 9 demonstrate that cells expressing a high level of wild-type HBGF-1 (Fig. 9 *B*) and to some extent a low level of wild-

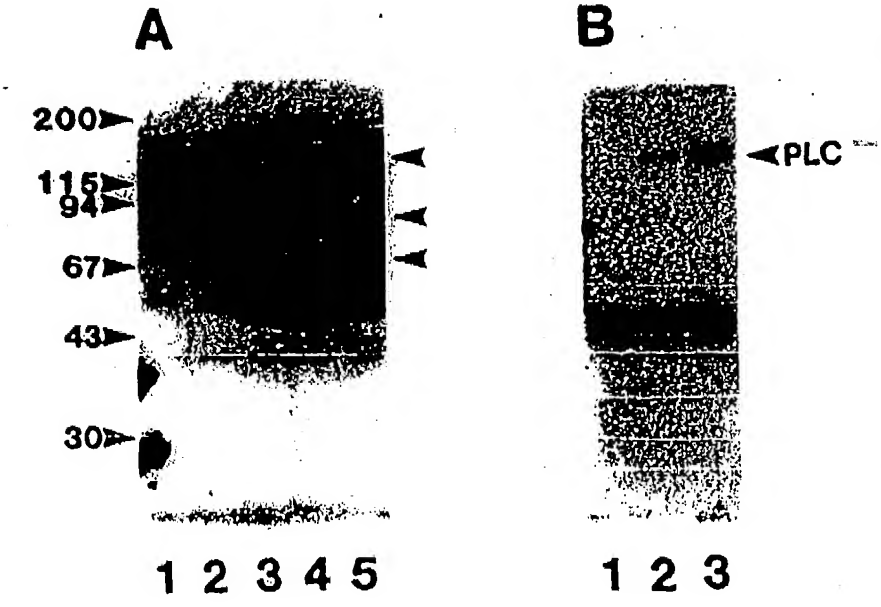

Figure 5. Stimulation of protein tyrosine kinase activity by wild-type and mutant HBGF-1. (*A*) Serum starved NIH 3T3 cells were either (lane *1*) unstimulated or treated with 5 U/ml heparin and (lane 2) 1 ng/ml wild-type; (lane *3*) 10 ng/ml wild-type; (lane *4*) 1 ng/ml mutant; or (lane *5*) 10 ng/ml mutant HBGF-1. The cells were processed as described in Materials and Methods and phosphotyrosine-containing proteins were visualized using antiphosphotyrosine antibodies and ^{125}I-protein A. The arrows indicate the positions of 150,000–, 90,000–, and 70,000–mol wt proteins whose phosphotyrosine content are increased by the addition of wild-type or mutant HBGF-1. (*B*) Cells were incubated as in *A* with the exception that cell lysates were immunoprecipitated with anti-phospholipase C-γ antibodies before Western blot analysis with anti-phosphotyrosine antibodies. Cells were either (lane *1*) unstimulated or treated with (lane 2) 10 ng/ml wild-type, or (lane *3*) 10 ng/ml mutant HBGF-1. The arrow shows the position of a 150,000–mol wt protein whose phosphotyrosine content is increased by treatment with wild-type or mutant HBGF-1.

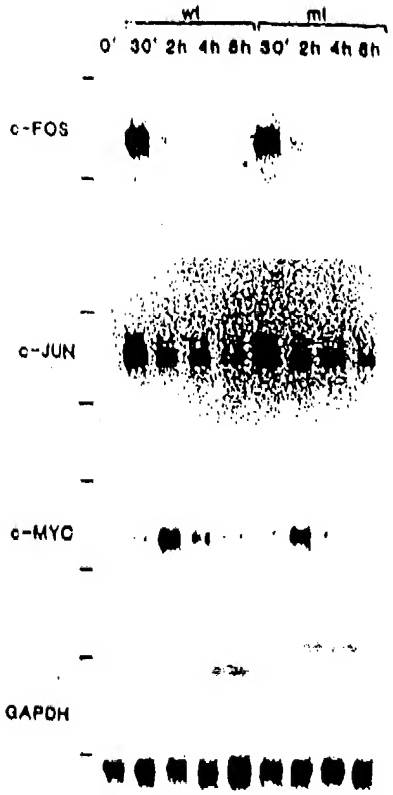

Figure 6. Effect of wild-type and mutant HBGF-1 on protooncogene mRNA levels. Serum starved NIH 3T3 cells were either left unstimulated or treated with heparin (5 U/ml) and 10 ng/ml wild-type (*wt*) or mutant (*mt*) HBGF-1 for the indicated time periods. RNA was prepared and used for RNA gel blot hybridization using the radiolabeled DNA probes indicated on the left side (*GAPDH*, glyceraldehyde 3-phosphate dehydrogenase). The upper and lower tick marks on the left side of each panel represent the positions of 28 and 18S rRNA, respectively.

type HBGF-1 (Fig. 9 *D*) have acquired a more polar, elongated phenotype characteristic of transformed 3T3 cells. This phenotype is not seen in cells expressing neomycin resistance alone (Fig. 9 *A*) or in cells expressing relatively high levels of mutant HBGF-1 (Fig. 9 *C*). It should be noted that we have not been able to detect HBGF-1 immunoreactivity in the media conditioned by these cells and that the cells expressing relatively high levels of wild-type HBGF-1 show enhanced growth in soft agar relative to untransfected cells or cells expressing high levels of the mutant HBGF-1 (data not shown). These results are consistent with the results of the mitogenic assays described above which demonstrate that the growth-promoting activity of the mutant HBGF-1 is relatively low when compared to the wild-type protein.

Discussion

The experiments described in this report were initiated as a result of the chemical modification studies of HBGF-1 reported by Harper and Lobb (19). They demonstrated that reductive methylation of HBGF-1 resulted in selective, stoichiometric modification of lysine residue 132 (using the 1–154 numbering system for full-length HBGF-1). It was suggested that modification of this residue, which is conserved in all HBGF-1 and HBGF-2 sequences reported to date, was responsible for the reduced apparent affinity for immobilized heparin, the reduced mitogenic capacity, and the reduced receptor-binding activity of the modified protein. The results presented here using site-directed mutagenesis to address the role of lysine 132 on the functional properties of HBGF-1 are in general agreement with the conclusions of Harper and Lobb (19). Specifically, substitution of lysine 132 for glutamic acid reduces the apparent affinity of the recombinant protein for immobilized heparin (elutes at 0.45 M NaCl compared with 1.1 M NaCl for wild-type) and significantly reduces the mitogenic potency of the growth factor. The reduced mitogenic potency may be a direct consequence of the reduced apparent affinity of the mutant HBGF-1 for heparin since it has been demonstrated that the class 1 heparin-binding growth factors in general (29) and human HBGF-1 in particular (22, 43) are dependent on the presence of heparin for optimal biological activity.

Our results do not support the notion that the reduced mitogenic capacity of HBGF-1 containing glutamic acid in place of lysine at position 132 is due to reduced binding to cell surface receptors. The receptor-binding properties of the mutant HBGF-1 are not distinguishable from those of the wild-type protein as judged by cross-linking experiments (see Fig. 4). In addition, the mutant HBGF-1 is able to induce the same pattern of tyrosine kinase phosphorylation as is the wild-type protein (see Fig. 5) and can induce protooncogene expression (see Fig. 6). The majority of the studies presented here utilize a heparin concentration of 5 U/ml; the concentration where maximal difference between the mitogenic activity of wild-type and mutant HBGF-1 was observed in the 3T3 cell thymidine incorporation assay. It should be noted that in the absence of heparin, the mutant HBGF-1 competes poorly with labeled wild-type HBGF-1 in cross-

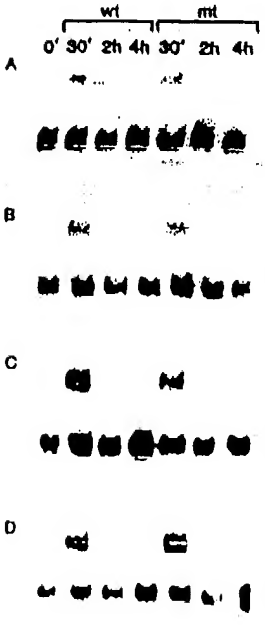

Figure 7. Effect of different concentrations of wild-type and mutant HBGF-1 on c-*fos* mRNA levels. Serum starved NIH 3T3 cells were either left unstimulated or treated with heparin (5 U/ml) and (*A*) 0.5 ng/ml, (*B*) 1.0 ng/ml, (*C*) 5.0 ng/ml, (*D*) 10 ng/ml wild-type (*wt*) or mutant (*mt*) HBGF-1 for the indicated time periods. RNA was prepared and used for RNA gel blot hybridization using the c-*fos* DNA probe (*upper panels*) or glyceraldehyde 3-phosphate dehydrogenase DNA probe (*lower panels*).

Figure 8. Western blot analysis of HBGF-1 in NIH 3T3 cells transfected with wild-type or mutant HBGF-1 expression plasmids. NIH 3T3 cells were transfected as described in Materials and Methods. The figure shows the relative levels of HBGF-1 immunoreactivity present in lysates of cells transfected with wild-type HBGF-1 (lane *1*, clone producing relatively high level of HBGF-1; lane *3*, clone producing relatively low level of HBGF-1) normal NIH 3T3 cells (lane 2), cells transfected with pSV2neo alone (lane *4*), and cells transfected with mutant HBGF-1 (lane *5*). For each cell type, 10^6 cells were lysed with 1 ml of 2× Laemmli sample buffer and a 60-μl aliquot was used in the Western blot.

linking assays (data not shown). In addition, whereas the apparent affinity of the mutant HBGF-1 for immobilized heparin is reduced, it does bind at ionic strengths (i.e., ~0.5 M NaCl) that exceed those known to be physiologic. Thus, the data presented here indicate that the mutant can utilize the presence of heparin to restore some (i.e., receptor-binding, tyrosine kinase activation, and protooncogene induction) but not all (i.e., stimulation of [^{3}H]thymidine incorporation into DNA and endothelial cell proliferation) of the activities of the wild-type protein. Similarly, it is of interest that the wild-type protein competes with labeled HBGF-1 for receptor-binding and induces protooncogene expression at similar concentrations in the presence or absence of added heparin yet it requires added heparin in order to promote DNA synthesis and cell proliferation (Figs. 1, 2, 4, and 6; and data not shown). Thus, the relatively poor mitogenic activity of the mutant protein may be related to its reduced apparent affinity for heparin. The data presented here demonstrate that "high" affinity receptor-binding, activation of tyrosine kinase activity, tyrosine phosphorylation of specific substrates, and induction of protooncogene expression may be necessary but are not, by themselves, sufficient to sustain a mitogenic response to the presence of HBGF-1. These results are consistent with the observations of Escobedo and Williams (12) who showed by site-directed mutagenesis of the PDGF receptor and cDNA transfection that mutants could be constructed that were responsive to PDGF with respect to receptor tyrosine kinase activation and increased phosphatidylinositol turnover but did not elicit a mitogenic re-

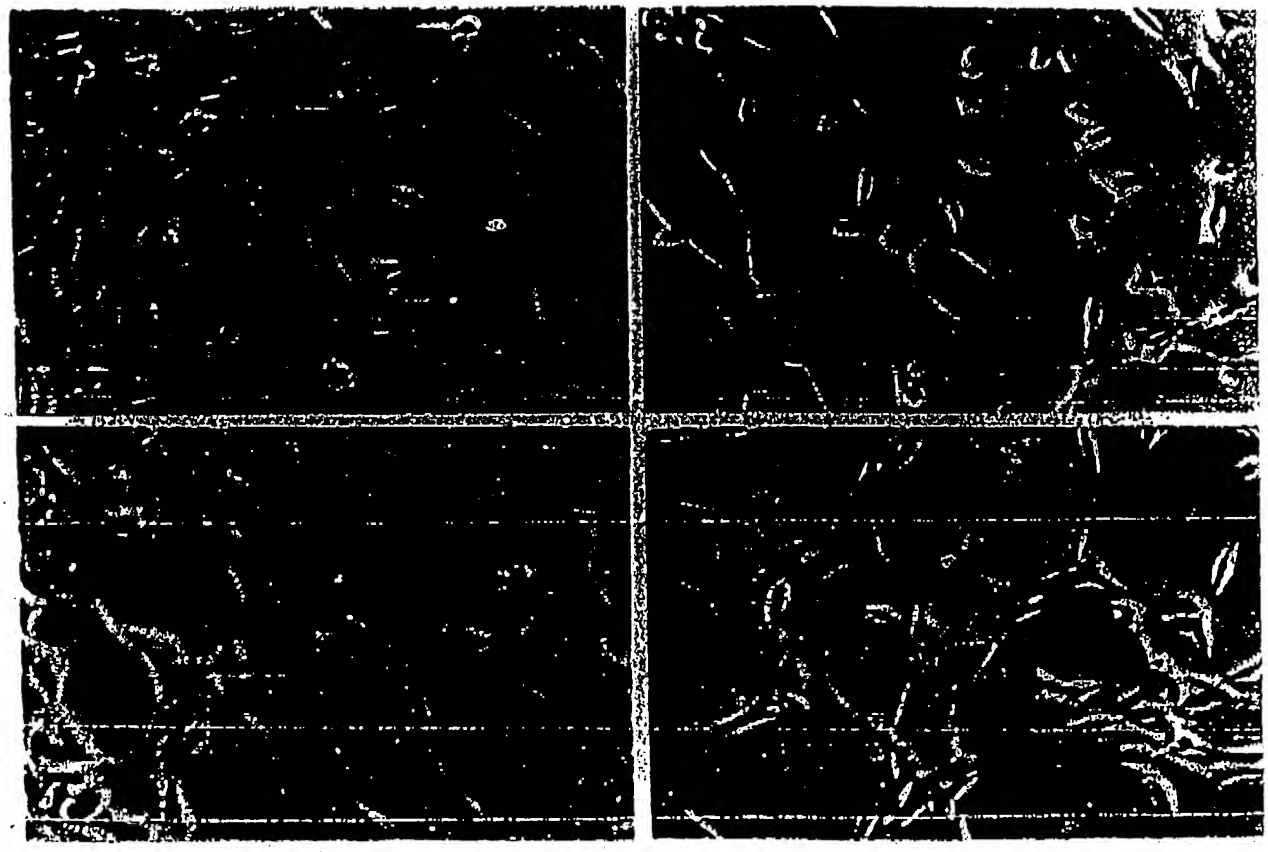

Figure 9. Morphology of NIH 3T3 cells transfected with wild-type or mutant HBGF-1 expression plasmids. The figure shows micrographs of the same NIH 3T3 cells analyzed by Western blot analysis in Fig. 8. *A* shows cells transfected with pSV2neo only and B–D show cells co-transfected with pSV2neo and expression vectors for wild-type (*B* and *D*) and mutant (*C*) HBGF-1. The cells shown in *B* correspond to those expressing relatively high levels of HBGF-1 (Fig. 8, lane *1*), whereas those shown in *D* correspond to those expressing relatively little HBGF-1 (Fig. 8, lane *3*).

sponse to PDGF. Similarly, Severinsson et al. (40) used similar methods to generate a system where the mutant receptor could mediate an increase in *c-fos* expression in response to PDGF but not actin reorganization or mitogenesis.

The mitogenic deficiencies of the mutant HBGF-1 may be due to reduced biological stability in tissue culture medium, reduced binding to cell surface proteoglycans, an altered intracellular stability, and/or an altered affinity for an intracellular receptor or binding protein. It has been established that the presence of heparin protects HBGF-1 from thermal and proteolytic inactivation (28, 37). In addition, it has been shown that ^{125}I-labeled HBGF-1 is relatively insensitive to lysosomal degradation after receptor-mediated endocytosis (14). There is no obvious difference in the susceptibility of wild-type and mutant HBGF-1 to proteolytic cleavage by the conditioned media of NIH 3T3 cells cultured in the presence of 10% calf serum. However, the relative resistance of wild-type and mutant HBGF-1 to proteolytic modification in the presence of target cells or after receptor-mediated endocytosis has not been established. It is also possible that the mutant protein is more susceptible than the wild type to nonproteolytic inactivation. Further studies should reveal whether the altered activities of the mutant HBGF-1 are a consequence of its reduced apparent affinity for heparin.

In summary, the data presented here demonstrate that the various functions of HBGF-1 can be dissociated at the structural level. The observation that site-directed mutagenesis can be used to produce recombinant proteins with "normal" receptor-binding activity and reduced mitogenic activity indicates that similar methods could be used to produce potent antagonists of HBGF-1. More importantly, these results indicate that it may be possible through structure–function analysis and site-directed mutagenesis to generate mutants that retain certain (i.e., chemotactic, mitogenic, or heparin-binding) but not other biological functions characteristic of the wild-type protein. Finally, whereas the data presented on the receptor-binding and tyrosine kinase activation properties of the p132E mutant demonstrate that a lysine residue at this position is not critical for these functions, it is still possible that methylation of a lysine at this position could lead to reduced receptor-binding activity of HBGF-1 (19).

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